ASSESSMENT OF COBALAMIN STATUS IN EXPERIMENTAL AND CLINICAL STUDIES BY INTRACELLULAR AND EXTRACELLULAR MARKERS OF VITAMIN FUNCTION

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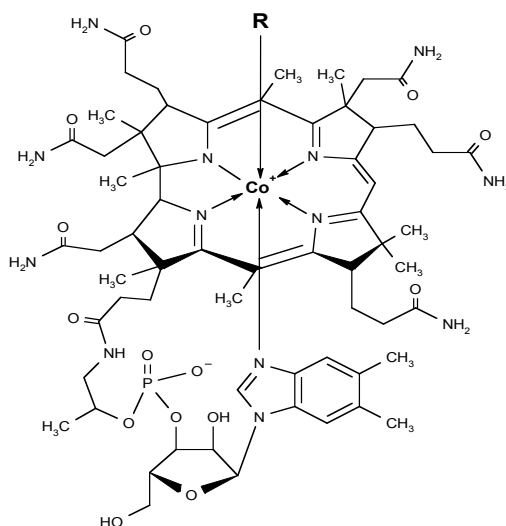Dissertation for the degree of doctor medicinae (dr.med.)

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Assessment of Cobalamin Status in Experimental and Clinical Studies by Intracellular and Extracellular Markers of Vitamin Function

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LIST OF PUBLICATIONS

This thesis is based on the following publications:

Paper 1   Riedel, B., Ueland, P.M., and Svardal A.M.
           Fully automated assay for cobalamin-dependent methylmalonyl-CoA mutase
           Clinical Chemistry (1995) 41:1164-1170

Paper 2   Riedel, B., Fiskerstrand, T., Refsum, H., and Ueland, P.M.
           Co-ordinate variations in methylmalonyl-CoA mutase and methionine
           synthase, and in the cobalamin cofactors in human glioma cells during nitrous
           oxide exposure and the subsequent recovery phase
           Biochemical Journal (1999) 341:133-138

Paper 3   Fiskerstrand, T., Riedel, B., Ueland, P.M., Seetharam, B., Pezacka E.H.,
           Gulati, S., Bose, S., Banerjee, R., Berge, R.K., and Refsum, H.
           Disruption of a regulatory system involving cobalamin distribution and
           function in a methionine-dependent human glioma cell line
           The Journal of Biological Chemistry (1998) 273:20180-20184

Paper 4   Riedel, B., Bjørke Monsen, A.L., Ueland, P.M., and Schneede, J.
           Effects of oral contraceptives and hormone replacement therapy on markers of
           cobalamin status
           Clinical Chemistry (2005) 51:778-781

ABBREVIATIONS

AdoCbl	5'deoxyadenosylcobalamin
AdoMet	S-adenosylmethionine
ATR	ATP:cobalamin adenosyltransferase
Cbl	cobalamin
Cbl(I,II,III)	mono-(I), di-(II), or tri(III)valent cobalamin
CNCbl	cyanocobalamin
CR	cobalamin reductase
HC	haptocorrin
Hcy	homocysteine
HoloTC	holo-transcobalamin
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
IF	intrinsic factor
MeCbl	methylcobalamin
MethylTHF	5-methyltetrahydrofolate
MCM	L-methylmalonyl-CoA mutase
Met	methionine
MMA	methylmalonic acid
MS	methionine synthase
MSR	methionine synthase reductase
MTHFR	5,10-methylenetetrahydrofolate reductase
N_2O	nitrous oxide
OC	oral contraceptives
OHCbl	hydroxycobalamin
TC	transcobalamin
TC-R	transcobalamin receptor
tHcy	total homocysteine
WB-folate	whole blood folate

BACKGROUND OF THE STUDY

History of cobalamin

The history of vitamin B_{12} (cobalamin, Cbl) originated with the descriptions of a progressive macrocytic anaemia, made independently by Combe, Addison, and Biermer in the nineteenth century. At that time, Lichtheim described also spinal cord lesions that were associated with haematological changes typically seen in macrocytic anaemia (see ([1]) for a review). However, it was not until Minot & Murphy ([2]) detected the anti-pernicious anaemia factor provided by crude liver homogenates and red meat in the diet that the role of this factor in haematological disorders became evident, and focus was directed towards its further identification. A research hunt spanning over 2 decades led independently to the isolation of the pure anti-pernicious anaemia factor, which dramatically changed the therapeutic solutions for pernicious anaemia from several grams of uncooked liver to a few micrograms of a red crystalline compound named vitamin B_{12} or Cbl ([3, 4]). In 1956 Hopkins resolved the complex structure of the Cbl molecule by X-ray crystallography ([5]), and a few years later the coenzyme function of the vitamin in bacterial glutamate metabolism was discovered in Barker's laboratory. They isolated and partly described an orange crystalline compound that was termed 5'deoxyadenosylcobalamin (AdoCbl) ([6]). The significance of AdoCbl as cofactor for methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) involved in propionate metabolism was demonstrated shortly after ([7, 8]). This was followed by the discovery of the other biologically active Cbl derivative, methylcobalamin (MeCbl), which was isolated both from liver and blood cells of humans and from bacteria ([9]). It was found to act as cofactor for mammalian methionine synthase (5-methyltetrahydrofolate homocysteine methyltransferase, MS, EC 2.1.1.13) in the methyl transfer reaction from homocysteine (Hcy) to form methionine (Met) ([10]). The structure of AdoCbl was exactly identified by X-ray analysis in 1966 ([11]), whereas the three-dimensional structure of MeCbl was first solved almost two decades later ([12]).
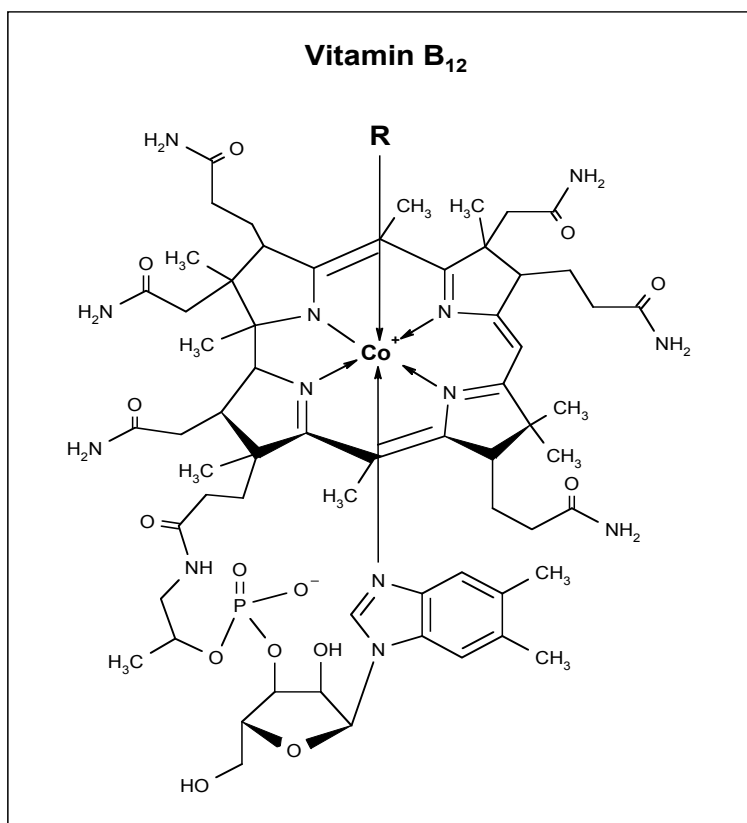
Figure 1. Structure of cobalamin.

The central cobalt atom has four nitrogen ligands from the corrin ring. R represents deoxyadenosine in adenosylcobalamin, methyl in methylcobalamin, -OH in hydroxycobalamin, and -CN in cyanocobalamin. The lower axial ligand is a nitrogen atom provided by dimethylbenzimidalzole.

Cobalamin chemistry

The vitamin is a water-soluble organo-metallic compound in which the central cobalt atom of a variable oxidation-reduction state is co-ordinated by four equatorial nitrogen ligands donated by pyrroles of a planar corrin ring. A nucleotide substituent, dimethylbenzimidazole, is bound to both the central cobalt atom and to the corrin ring (Figure 1). Different upper axial ligands may be attached to the central cobalt atom giving MeCbl, AdoCbl, hydroxycobalamin (OHCbl), or cyanocobalamin (CNCbl) (Figure 2) (5). Only MeCbl and AdoCbl are known to act as specific coenzymes in mammalian systems, while OHCbl is a naturally occurring Cbl precursor during coenzyme synthesis. CNCbl or OHCbl are usually used for supplementation purpose. In OHCbl and CNCbl, the central cobalt atom is in the trivalent (Cbl(III)) state, and

reduction to divalent (Cbl(II)) and monovalent Cbl (Cbl(I)) is required for the synthesis of both AdoCbl (13) and MeCbl (14).

Cbl is of bacterial origin (3) and is widely distributed in animal tissues (15). It cannot be synthesized by man and has to be supplied through the diet, either derived from bacterially fermented food or from animals that ingest and store bacterial Cbl (16).

Metabolic role of cobalamin

In mammals, Cbl acts as coenzyme in two enzyme reactions: mitochondrial MCM, which requires AdoCbl, and cytosolic MS, which requires MeCbl as cofactor.

AdoCbl-dependent MCM catalyzes the reversible interconversion of L-methylmalonyl-CoA to succinyl-CoA as the final step in propionyl-CoA metabolism. Propionyl-CoA is formed in animal tissue by the degradation of odd-chain fatty acids, cholesterol, and certain essential amino acids, including valine, isoleucine, and methionine (17). The product of the reaction, succinyl-CoA, enters the citric acid cycle (Figure 2).

MS is a key enzyme in one-carbon metabolism (18). It catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate (methylTHF) to Hcy via MeCbl to give tetrahydrofolate (THF) and Met (19) (Figure 2). In this reaction, Cbl is transiently converted to MeCbl, as it accepts the methyl group from methylTHF and delivers it to the substrate, Hcy (20). This latter reaction represents the intersection between Cbl and folate metabolism (Figure 2).

Met is incorporated into proteins or, via its activated form, S-adenosylmethionine (AdoMet), is the principal substrate for numerous methylation reactions involving DNA, RNA, proteins and phospholipids (Figure 2). The other product of the reaction, THF, is required for the formation of reduced folates, like 5,10-methylenetetrahydrofolate and 10-formyltetrahydrofolate used in the synthesis of thymidylate and purines for DNA formation (21).

Hcy metabolism also involves other B-vitamins. Vitamin B_2 functions as cofactor for methylenetetrahydrofolate reductase (EC 1.5.1.2.0) for the conversion from 5,10-methylenetetrahydrofolate to methylTHF. Cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.11) are vitamin B_6-dependent enzymes required for the ultimate metabolism of Hcy in the transsulfuration pathway (Figure 2).
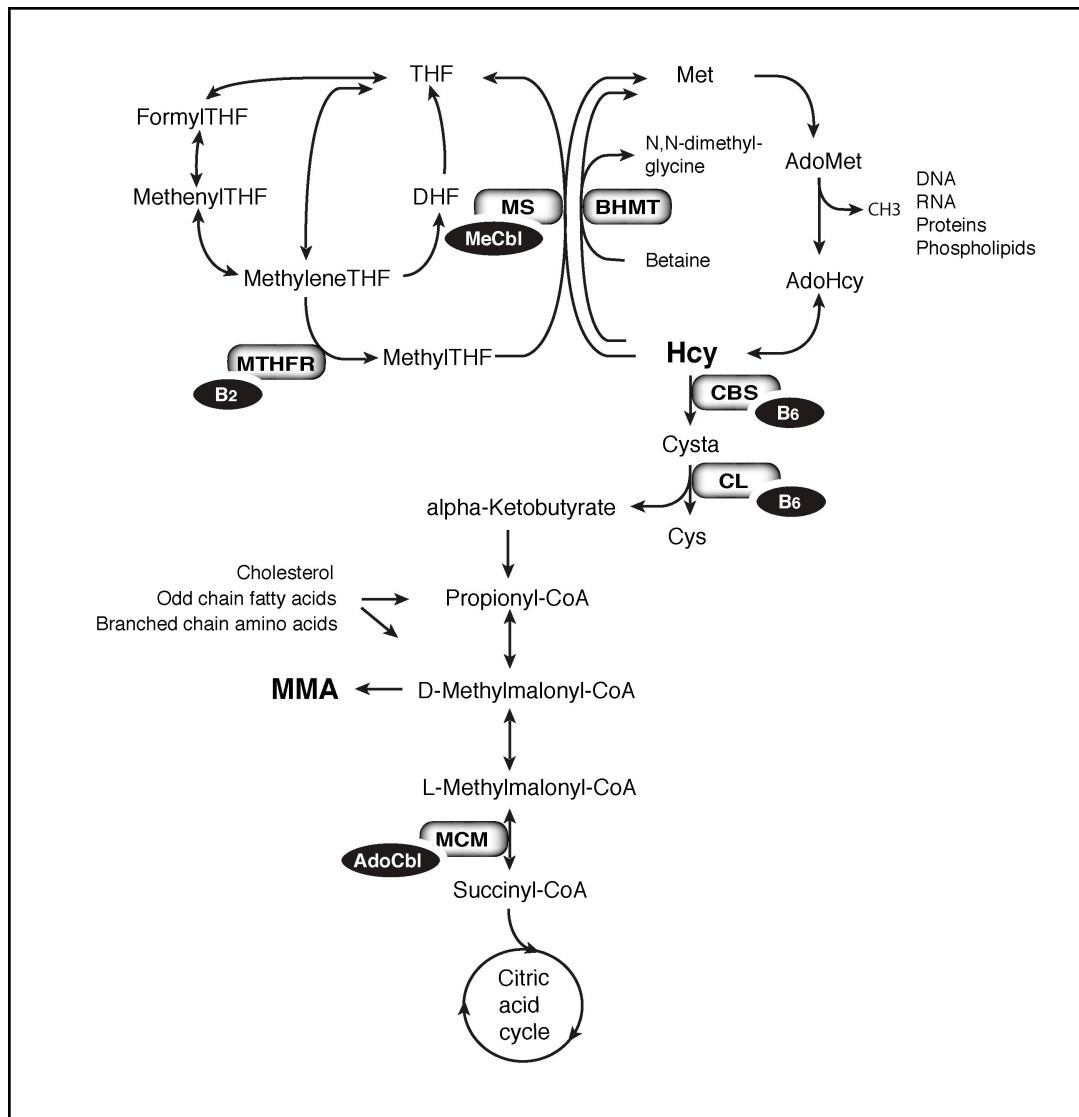
Figure 2. B vitamins and enzymes in homocysteine and methylmalonic acid metabolism. Homocysteine (Hcy) is formed from S-adenosylhomocysteine (AdoHcy), and may be salvaged to methionine (Met) by methylcobalamin (MeCbl)-dependent MS, which requires 5-methyltetrahydrofolate (MethylTHF) as substrate, or by betaine-homocysteine methyltransferase (BMHT), which uses betaine as the methyl-donor. MethylTHF is derived from 5,10-methylenetetrahydrofolate (MethyleneTHF) by the action of vitamin B_2-dependent methylenetetrahydrofolate reductase (MTHFR). Hcy is ultimately degraded through the transsulfuration pathway, which involves the conversion of Hcy to cysteine (Cys) by two vitamin B_6-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CL). Methylmalonic acid (MMA) is derived from D-methylmalonyl-CoA in the propionyl-CoA pathway. D-methylmalonyl-CoA is predominantly metabolized to succinyl-CoA via L-methylmalonyl-CoA by the catalytic action of 5'deoxyadenosylcobalamin (AdoCbl)-dependent methylmalonyl-CoA mutase (MCM). AdoMet, S adenosylmethionine; Cysta, cystathionine; DHF, dihydrofolate; FormylTHF, 10-formyltetrahydrofolate; THF, tetrahydrofolate.

Cobalamin transport and metabolism

Intestinal transport and uptake

Cbl is released from dietary proteins by peptic digestion within the acidic environment of the stomach ([22](#)), and it becomes initially bound to salivary and gastric derived R-binder or haptocorrin (HC). This transport protein is synthesized in a variety of cells and tissues, including exocrine glands, gastric mucosa, myeloid cells and liver cells. It is present in many secretions and extracellular fluids, such as saliva, plasma and bile ([23](#)).

HC is digested by pancreatic proteases in the duodenum, which allows the binding of liberated Cbl to intrinsic factor (IF), produced in gastric parietal cells. IF is critical for intestinal Cbl absorption. Disorders associated with absent or dysfunctional IF may cause megaloblastic anaemia, the classical pernicious anaemia, and neurological disturbances (([24](#)) and references cited therein). These disorders are also related to developmental delay and growth retardation if the disease commences in early childhood ([25](#)).

The site of Cbl absorption in man is the distal small intestine ([26](#)), where the IF-Cbl complex is internalized into the enterocyte by a Ca^{2+}-dependent process ([27](#)) in cooperation with the multiligand receptor, cubilin-amnionless ([28, 29](#)). Impaired expression or function of the IF-Cbl receptor, cubilin, because of mutations in the receptor-coding gene, leads to decreased uptake of the IF-Cbl complex and functional Cbl deficiency (Gräsbeck-Imerslund disease) ([30-32](#)).

Cbl absorbed in the intestine becomes bound to transcobalamin (TC) and subsequently enters the liver via the portal system. Cbl bound to HC in blood may be excreted in the bile, and Cbl is reabsorbed in the ileum by IF-mediated transport. This enterohepatic circulation is an effective way of Cbl conservation as 3-5 times more Cbl is reabsorbed than is newly taken up from food ([16](#)).

There is evidence that also unbound Cbl can pass cell membranes if the concentration of unbound vitamin is increased to sufficient high concentrations. This process is Ca^{2+}-independent and sensitive to inhibitors of protein synthesis ([33](#)). However, the functional role of this uptake process, under most physiological circumstances, is probably of little significance.

Dietary Cbl
1 — Cbl bound to food proteins

Liver

Gastric ventricle

2

7

8 — Bile

Pancreas

10

3

Kidney
Re-absorption of TC-bound Cbl in renal proximal tubules by inter-action of TC-R with megalin

Distal ileum

4 — Aborption of TC-bound Cbl via cubilin receptor in ileal mucosal cells

9

Blood circulation

5 — Cbl transport in blood bound to TC and HC

6

TC-R mediated uptake of TC-bound Cbl in peripheral cells

Homocysteine → Methionine

MMA-CoA → Succinyl-CoA

Legend:
- Cbl, Cobalamin
- HC, Haptocorrin
- TC-R, TC-receptor
- Cubilin
- Intrinsic factor
- TC, Transcobalamin
- Cbl bound on enzyme
- Megalin

18

Figure 3. Cobalamin absorption and metabolism.

Dietary cobalamin (vitamin B^{12}, Cbl) is normally protein-bound **(1)** and provided by food products of animal origin. Pepsin and low pH in the gastric ventricle degrade food proteins, resulting in release of Cbl **(2)**. Free Cbl is then bound to R-binder or haptocorrin (HC), which is produced by salivary glands and parietal cells. HC is degraded by pancreas proteases, and Cbl (both newly ingested and Cbl bound to HC in the bile) is released again and binds with high affinity to intrinsic factor (IF) produced in the stomach **(3)**. Cbl has high affinity to IF at neutral or alkaline pH of the pancreatic juice. In the mucosal cells of the distal 80 cm of the ileum, the Cbl–IF complex is recognized by cubilin receptor **(4)**. Cbl enters the blood circulation bound to transcobalamin (TC). There, the majority of Cbl (70-80%) is bound to HC and only a minor portion (20-30%) is bound to TC **(5)**. TC-bound Cbl (holoTC) is the biologically active fraction of total Cbl in serum, as only this fraction is taken up by most cells in the body. Cellular uptake of holoTC is mediated by TC-receptors **(6)**. Cbl absorbed in the intestine becomes bound to TC and subsequently enters the liver **(7)** via the portal system **(10)**. Within the cells, holoTC-molecules are degraded and Cbl is enzymatically converted into its two coenzyme forms, methylCbl (cofactor for methionine synthase) and adenosylCbl (cofactor for methylmalonyl-CoA mutase) **(6)**. There is extensive enterohepatic circulation transporting 3-5 times more Cbl than is newly absorbed from food. Cbl and Cbl analogues are bound to HC in the bile **(8)**. The kidneys seem to have a more important role for Cbl homeostasis than earlier recognized. HoloTC is filtered in the glomeruli and quantitatively reabsorbed in the proximal tubules in a process involving the TC-receptor and megalin **(9)**. Cbl leaves tubules cells at the basal membrane bound to TC.

Transport in plasma

IF is degraded in the enterocyte lysosomes, and Cbl is released into the portal circulation bound to transcobalamin (TC). TC transports newly absorbed Cbl to the liver ([34]), and from its storage sites, i.e. the liver, to the tissues ([35]). TC is derived from a variety of cells and tissues, including enterocytes, hepatocytes, renal cells and endothelial cells, and can be synthesized by a number of cultured cells, like fibroblasts, macrophages and human bone marrow cells ([36]).

In plasma, most of the Cbl is bound to HC, and only a minor fraction, < 30 %, is bound to TC. Only TC-bound Cbl is available for cellular uptake by receptor-mediated transport involving a specific receptor for TC, TC-R ([37, 38]). Decreased or no synthesis of functional TC (TC-deficiency) due to mutations in the TC gene is associated with functional Cbl deficiency ([39-41]). In contrast, the inherited deficiency of HC is solely accompanied by low serum Cbl that does not lead to tissue deficiency ([42]). Several base pair substitutions may occur in the gene that encodes for TC, and these may affect protein structure and function ([43,

44). The most commonly occurring 776C>G polymorphism probably affects the binding affinity for Cbl and the ability to transport Cbl into tissues (44-46). Some (44, 47, 48), but not all studies (49, 50) report an association between this polymorphism and increased concentrations of plasma MMA, plasma Hcy, or decreased concentrations of TC-bound Cbl in plasma.

Cellular uptake, intracellular transport and cofactor formation

TC-bound Cbl, denoted holoTC, complexes with TC-R on the cell surface and is internalized by endocytosis (38). In a process involving the multiligand-receptor, megalin, TC-R also seems to be important for tubular reabsorption of holoTC filtrated through the glomeruli of the kidney (51).

Although intracellular Cbl trafficking involves still incompletely understood reactions, many studies, especially those related to the discovery and descriptions of the inherited disorders in intracellular Cbl metabolism, have revealed important insight into the mechanisms of Cbl cofactor synthesis.

The internalized TC-R-bound TC-Cbl complex is brought to the lysosomes where it dissociates (52). The TC-moiety is degraded by lysosomal proteases (53), and free Cbl is transported into the cytosol (54), where it is subjected to an exchange of the upper axial ligand by action of β-ligand transferase (55, 56). As the oxidation-reduction state of the cobalt atom in the Cbl molecule is a critical factor for its function, Cbl(III) has to be reduced to Cbl(II) and subsequently to Cbl(I) (57). In bacteria, a NADH-dependent Cbl(III) reductase (CR) (EC 1.6.99.8) has been described (13, 58), and similar enzymes are located in the microsomes and the outer mitochondrial membrane of both rat and human liver cells (59, 60). These NADH- or NADPH-dependent enzymes have been shown to be involved in coenzyme synthesis, and probably reduce Cbl(III) to Cbl(II) (56, 60-62).

Inborn errors probably related to either a defective removal of the upper axial ligand or to a defective intracellular Cbl reductive system or both, termed *CblC* and *CblD*, or to impaired lysosomal Cbl release (*CblF*), may affect the synthesis of both AdoCbl and MeCbl, and subsequently reduce the activities of their respective enzymes, leading to combined homocystinuria and methylmalonic aciduria (17). Recently, a *CblD* defect has been described that causes either isolated or combined deficiency of MeCbl and AdoCbl synthesis, which points to further complexity of intracellular Cbl metabolism (63). Neither of these enzymes has been purified, and only recently the gene for the *CblC* defect has been cloned (64).

Cbl(II) becomes associated with cytosolic MS ([57]), and is reductively methylated to MeCbl during catalytic action of the enzyme ([14, 65]). During the catalytic cycle, the Cbl alternates between Cbl(I) and the active MeCbl. However, Cbl(I) is a strong reductant and becomes occasionally oxidized to Cbl(II), rendering the enzyme inactive. Regeneration of functional enzyme requires reductive methylation catalyzed by the flavoenzyme, methionine synthase reductase (MSR), which utilizes AdoMet as the methyl donor ([66, 67]). Mutations in the MS and the MSR genes, respectively, termed *CblG* and *CblE*, impede the formation of MeCbl, leading to low catalytic MS activity and causing isolated homocystinuria ([68]).

The cellular metabolism of Cbl located to steps after lysosomal hydrolysis of TC and before the binding of the Cbl coenzymes to their respective apoenzymes has not been fully elucidated. It is not known which Cbl form is exchanged across the mitochondrial membrane. The first reductive step, the conversion of Cbl(III) to Cbl(II), may occur in the cytosol. This idea is supported by the observation that patients with *CblC* disease, who are assumed to have impaired cytosolic CR activity, have increased levels of tHcy and MMA and low content of both AdoCbl and MeCbl ([17, 68]). However, Watanabe and colleagues described a partial deficiency of a NADH-dependent CR located in the mitochondrial fraction of *CblC* fibroblasts ([62]). Thus, whether Cbl(II) or Cbl(III) penetrates the mitochondrial membrane, and whether Cbl(II) can leave mitochondria and enter the cytoplasm of cells for use in MeCbl synthesis, is unknown.

In the mitochondria, Cbl(II) undergoes an additional reduction to Cbl(I) which is a prerequisite for the final AdoCbl synthesis. Recent spectroscopic data indicate that Cbl(II) binds to ATP:cobalamin adenosyltransferase (ATR) (EC 2.5.1.17) which, in the presence of ATP, initiates the reduction of Cbl(II) to Cbl(I) and catalyzes the adenosyl-group transfer of ATP to Cbl(I) to form AdoCbl ([69]). The necessary physiologic reductant for this reaction is not known. The gene encoding ATR, denoted MMAB, has been identified and cloned recently ([70, 71]). Another newly identified gene in humans, denoted MMAA, is a protein with GTPase activity but of otherwise unknown function ([72]). Mutations in both MMAB and MMAA, impede AdoCbl formation and are linked to the *CblB,* and *CblA* complementation group, respectively. They all result in isolated methylmalonic aciduria. Isolated methylmalonic aciduria is also caused by defects of the MCM apoenzyme, leading to either no (mut^0) or to decreased (mut^-) enzyme activity with reduced affinity for AdoCbl ([17]).
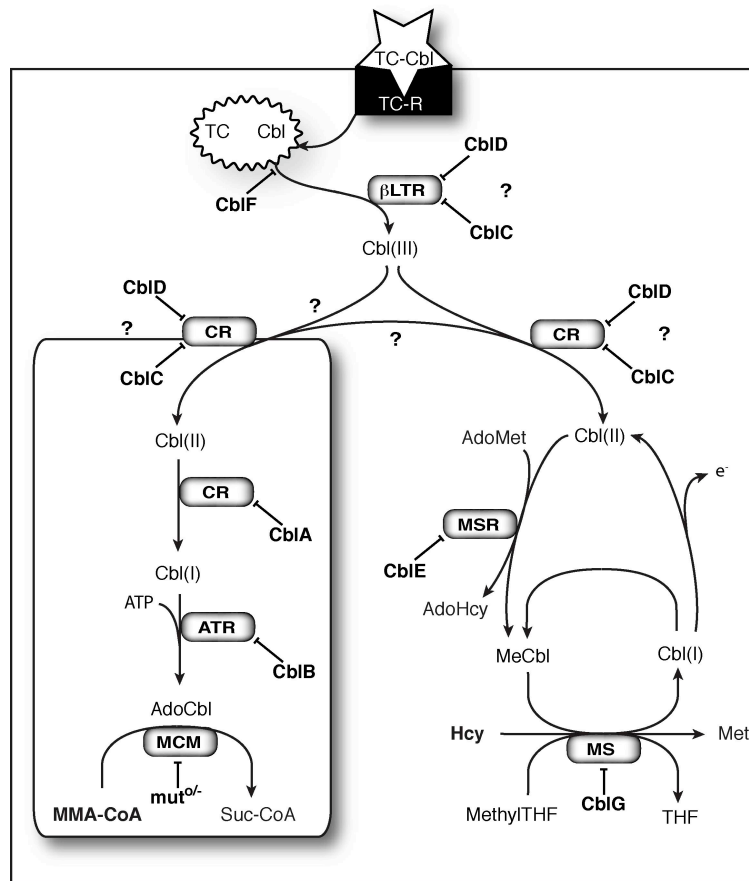
Figure 4. Genetic defects of intracellular cobalamin processing.

Following transcobalamin receptor (TC-R)-mediated uptake and lysosomal digestion of the transcobalamin-cobalamin (TC-Cbl) complex, Cbl is released into the cytosol, a process impaired in *CblF* mutants. The exact sites of the errors associated with *CblC* and *CblD* mutants are not definitely proven. They are presumably linked to β-ligand transferase (βLTR) and/or Cbl reductase (CR) located in the cytosol and probably in the outer mitochondrial membrane, leading to impaired removal of the upper axial ligand and insufficient reduction of trivalent Cbl (Cbl(III)) to divalent Cbl (Cbl(II)), respectively. Deficient formation of adenosylCbl (AdoCbl) can be caused by one of two distinct genetic enzyme defects, either related to mitochondrial CR, termed *CblA*, or to ATP:cobalamin adenosyltransferase (ATR), termed *CblB*. The defects located in the gene for methylmalonyl-CoA mutase (MCM) are associated with no (mut^0) or reduced (mut^-) catalytic enzyme activity, both of which cause methylmalonic aciduria. The *CblE* and *G* mutants, exclusively leading to reduced methylcobalamin (MeCbl) formation and homocystinuria, are caused by impaired reductive activation of methionine synthase (MS) and reduced remethylation of homocysteine (Hcy) to methionine (Met) due to mutations in the genes for methionine synthase reductase (MSR) and MS, respectively. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; MethylTHF, 5-methyltetrahydrofolate; MMA-CoA, L-methylmalonyl-CoA; Suc-CoA, succinyl-CoA; THF, tetrahydrofolate.

Intracellular cobalamin distribution

Virtually all intracellular Cbl is protein-bound, either to MS and MCM, the main intracellular Cbl-binding proteins ([73-75]), to ATR ([69]), and possibly to other enzymes involved in intracellular Cbl trafficking. The concentrations of the different intracellular Cbl forms have been measured in a variety of mammalian cell lines, including normal and mutant fibroblasts ([75]), various cancer cells ([76, 77]) and haematopoietic cells ([78, 79]). Internalized CNCbl is converted to OHCbl, AdoCbl and MeCbl ([33, 80]). AdoCbl has been shown to be the predominant Cbl derivative in rat and human liver cells and in haematopoietic cells ([78, 79, 81]), whereas in normal human skin fibroblasts most of the internalized Cbl is converted to MeCbl rather than to AdoCbl ([55]).

Accumulation and distribution of the naturally occurring Cbl derivatives, may differ markedly between tissues ([78]), probably due to a variety of factors, including alterations in cell proliferation and folate status ([79, 82]), or the cells' ability to remethylate homocysteine ([76, 77]). In addition, tissue distribution is affected by inherited defects in intracellular Cbl trafficking ([55, 75, 83, 84]). Whether common genetic polymorphisms in genes related to MCM and MS also may influence intracellular Cbl concentrations, is not known.

The two variants of the GaMg human glioma cells, P60 and P60H, used in this thesis have been characterized with respect to the concentrations and the distribution pattern of the different intracellular Cbl forms. Although the absolute concentrations may vary, the relative amounts of the intracellular Cbls were rather stable during standard culture conditions in Met medium. Met-dependent P60 cells contained high concentrations of CNCbl and OHCbl, but only very low levels of AdoCbl and MeCbl. In Met-independent P60H cells, the Cbl distribution pattern was characterized by high level of all four Cbl derivatives that were analyzed, although OHCbl and CNCbl were the predominant forms. In both cell variants, the concentration of AdoCbl was generally slightly higher than the concentration of MeCbl (Figure 7).

Cobalamin–dependent enzymes

Methylmalonyl-CoA mutase

AdoCbl-dependent MCM has been found in most mammalian tissues ([15]). It is a mitochondrial matrix enzyme ([85]) that is synthesized in the cytosol as a precursor, imported

into the mitochondrion and processed post-translationally to form active enzyme ([86]). Human MCM has been cloned ([87, 88]), and is encoded by the *MUT* nuclear gene that has been mapped to chromosome 6p21 ([89, 90]). Multiple mutations within the gene have been described. They are classified as being mut^0 (enzyme activity not detectable), or mut$^-$ (enzyme activity is detectable) (se Ref. ([91]) and references therein), but their significance for Cbl function and MCM activity is not clear, and no association with neural tube defects has been found ([92]).

Mammalian MCM is a functional homodimer of identical 77.5 kDa subunits, each with an active binding site for one AdoCbl molecule. The human enzyme binds its cofactor tightly, which both increases enzyme stability, and prevents AdoCbl from photolytic degradation. Purified enzyme from mammalian kidney and liver has been reported to be largely saturated with coenzyme, i.e. is present as holoenzyme ([93-95]). Less than 50 % of the purified enzyme from human placenta has bound AdoCbl ([96]), and in crude cell or tissue homogenates, only a minor fraction, 1 to 25 %, of MCM contains covalently bound AdoCbl. This has been shown in human fibroblasts, mouse and rat liver, and baby hamster kidney cells ([94, 97-99]), and indicates that the enzyme in these tissues is largely in the apoenzyme form.

Only MCM with bound AdoCbl, denoted holoMCM, is catalytically active. The reminder, denoted apoMCM, may in part be converted to holoMCM in a concentration- and time-dependent manner when Cbl is added to the cell culture medium ([55, 98-100]). In normal fibroblasts, the increase in the catalytic MCM activity during Cbl supplementation was not accompanied by any increase in propionyl-CoA pathway activity, which may suggest that, under Cbl sufficient conditions, MCM is not the rate-limiting enzyme in propionyl-CoA metabolism ([100]).

Addition of AdoCbl to crude tissue extracts in vitro converts all available apoMCM to holoMCM, and is as such a measure of total enzyme activity. ApoMCM activity can be calculated by subtraction of the amount of holoMCM activity from the amount of total MCM activity.

Methionine synthase

MS is widely distributed in cells and tissues, and within the cell, the enzyme is located in the cytoplasm. The enzyme has been purified from bacteria ([101]) and mammalian tissues, such as rat liver ([102]), porcine liver ([103, 104]) and human placenta ([105]). Human MS has been cloned and mapped to chromosomal location 1q43, and has a predicted molecular mass of

140-141kD ([106-108](#)). The first mutations have been found by sequencing cDNAs from patients with symptoms and signs indicative of a defect in the MS gene assigned to the *CblG* group ([107, 109, 110](#)). In addition, several polymorphisms are reported to date (reviewed in ([111](#)), one of which, the 2756A>G SNP, has been shown by some ([112-114](#)), but not all investigators ([115](#)) to be a genetic determinant of plasma tHcy.

The enzyme functions as a monomer with one active binding site for Cbl ([105](#)). MS is predominantly saturated with Cbl, as has been demonstrated under strictly anaerobic conditions ([116](#)). Like the bacterial enzyme, the mammalian MS binds Cbl tightly ([116, 117](#)), and binding of cofactor has been shown to stabilize the apoenzyme, which as such may be a regulatory mechanism ([118](#)). Studies of bacterial MS indicate that both MeCbl and Cbl(II) are bound to the enzyme ([119](#)), and Cbl(III) must be reduced to Cbl(II) before binding to the enzyme can occur ([57](#)). Also free MeCbl cannot bind to MS, but is generated on the enzyme during catalytic activity ([57, 120](#)).

Transcobalamin receptor

Circulatory Cbl bound to plasma TC (holoTC) is taken up by receptor-mediated endocytosis via TC-R ([38](#)). The functional importance of this uptake mechanism has been underscored by the observation that subjects with hereditary TC deficiency were severely Cbl-deficient ([24](#)), and rabbits, producing antibodies to human TC-R, developed Cbl deficiency caused by antibody-mediated inactivation of the receptor in many tissues ([121](#)). Thus, TC-R is a critical cell surface membrane protein that regulates intracellular utilization of Cbl.

The gene for TC-R has not yet been cloned. TC-R is a glycosylated protein with a monomeric molecular mass of 62 kDa ([122](#)) or 58 kDa ([123](#)) that is expressed as a functional homodimer in cellular plasma membranes. The receptor has been demonstrated on and isolated from human placental trophoblasts and hepatocytes ([124, 125](#)), and holoTC binds to TC-R ([124](#)). Cycloheximide, a protein synthesis inhibitor, caused a gradual decrease in receptor-specific uptake of Cbl, and an estimated receptor half-life was about 8 hours ([53](#)).

Cobalamin deficiency

Metabolic consequences

The enzyme activities of MS and MCM are dependent on cofactor availability. Insufficient cellular delivery of the vitamin, disturbed cofactor formation or impaired utilisation may result in decreased enzyme catalysis. Inherited abnormalities in intracellular Cbl metabolism that have been described to involve a decrease in the intracellular formation of AdoCbl and MeCbl are associated with decreased specific activities of MCM and MS ([17]). The decreased enzyme activities in fibroblasts from patients with these disorders can be stimulated in vitro by incubation with Cbl, and patients may be treated successfully with high doses of Cbl ([17]). Also in experimentally induced Cbl deficiency, the catalytic activity of the two Cbl-dependent enzymes is decreased. For MS, this has been shown with isolated enzyme ([126]), in cultured cells ([127-129]), and for both MS and MCM in different tissues from Cbl-deficient animals, including rats ([81, 130, 131]), sheep ([132]) and cattle ([133]).
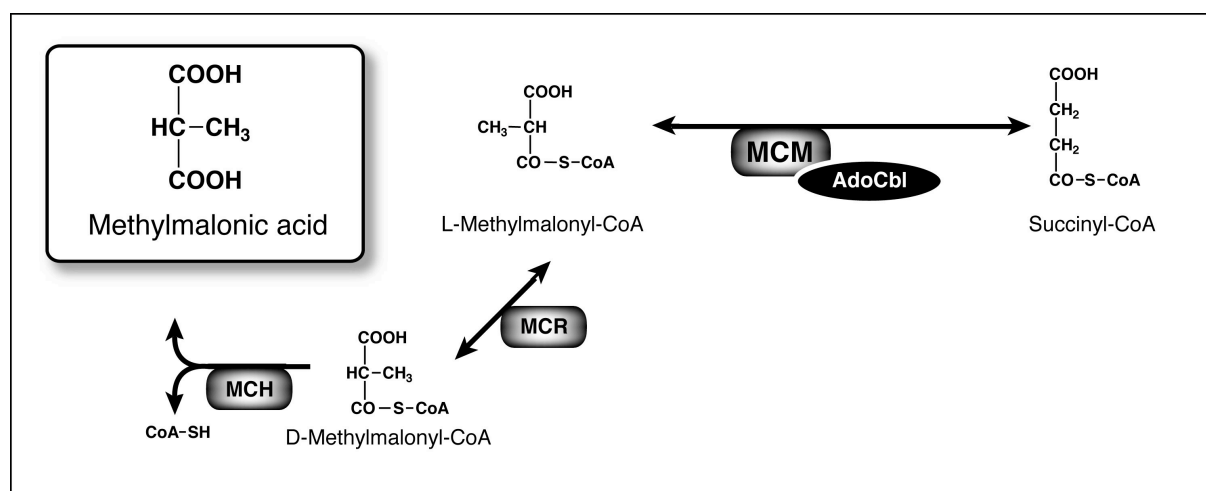
Figure 5. The Cbl-dependent enzyme, L-methylmalonyl-CoA mutase involved in methylmalonic acid metabolism. AdoCbl, 5'deoxyadenosylcobalamin; MCM, L-methylmalonyl-CoA mutase; MCR, D,L-methylmalonyl-CoA racemase, MCH, D-methylmalonyl-CoA hydrolase.

Decreased catalytic MCM activity may impede synthesis of succinyl-CoA from L-methylmalonyl-CoA, which then accumulates and, in a reversible reaction, is converted to D-

methylmalonyl-CoA by D,L-methylmalonyl-CoA racemase. D-methylmalonyl-CoA in turn is metabolized to MMA by D-methylmalonyl-CoA hydrolase (Figure 5). The subsequently increased concentration of MMA in cell culture medium (134), urine (131, 135, 136), plasma (137), or cerebrospinal fluid (138, 139) is an indicator of functional Cbl deficiency.

During Cbl depletion, MCM apoenzyme activity is increased in rat and pig liver (130, 140), unchanged in liver of sheep (132), cattle (133) and rat (81), or even reduced in rat liver in another study (131). Also, the specific activities of rat liver NADH and NADPH linked CR involved in cofactor synthesis are increased in Cbl-deficient animals compared to control animals (61).

The perturbation of propionate metabolism caused by decreased flux through MCM leads to accumulation of MMA and is eventually associated with chemical abnormalities such as metabolic acidosis, hyperglycinaemia, ketonaemia and/or hyperammonaemia (17), and with clinical signs of neuronal damage (141).
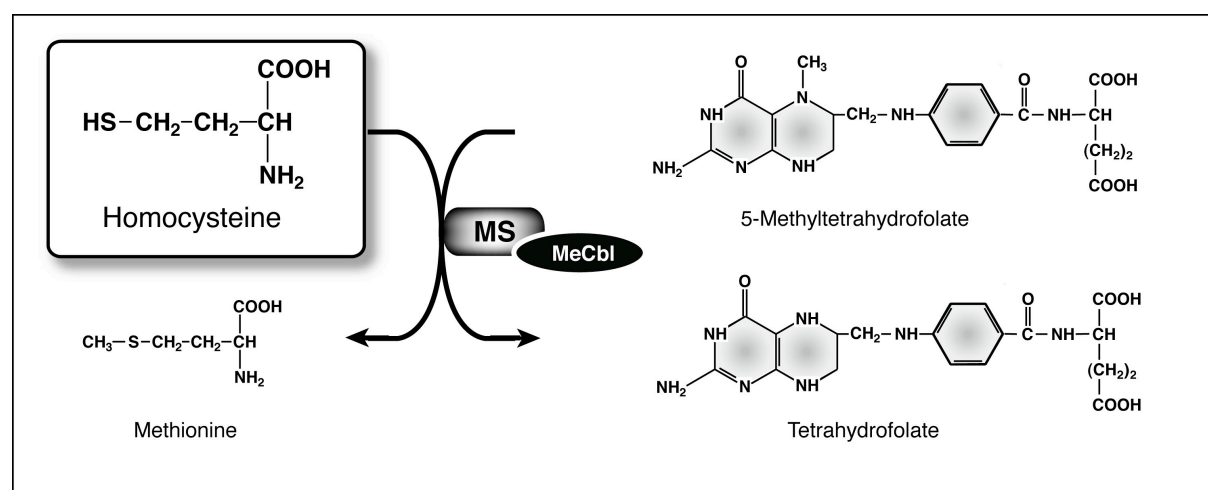
Figure 6. The Cbl-dependent enzyme, methionine synthase, responsible for the remethylation of homocysteine to methionine. MeCbl, methylcobalamin; MS, methionine synthase.

Cbl deficiency decreases the catalytic activity of MS, which impedes the formation Met and THF, the two products of the MS reaction (Figure 6). Met is incorporated into proteins or, via its activated form, AdoMet, is the principal substrate for methylation of DNA, RNA, proteins and phospholipids. Impaired transmethylation may be part of the pathogenesis of the

neuropathy associated with Cbl deficiency ([142](#)). THF is required for the formation of reduced folates, like 5,10-methylene-THF and 10-formyl-THF used in the synthesis of thymidylate and purines for DNA formation ([21](#)) (Figure 1). Perturbation of folate metabolism through impaired formation of THF may be responsible for the haematological changes seen in Cbl-deficient patients.

Accumulation of Hcy in cell culture medium ([128, 143](#)), plasma ([144-146](#)), urine ([147, 148](#)) or cerebrospinal fluid ([149](#)) may indicate Cbl or folate-deficiency.

Clinical implications

Intracellular Cbl deficiency results in multi organ disorders that typically include haematological abnormalities, such as anaemia, macrocytosis and hypersegmented neutrophils, and neurological symptoms related to demyelination in the central or peripheral nervous system ([150](#)).

However, Cbl deficiency is a heterogeneous state that may not always be associated with typical haematological and neurological abnormalities ([150](#)). Tissue Cbl deficiency without involvement of bone marrow or nervous system has been shown to be common ([144, 151, 152](#)). Low or marginal Cbl status has been associated with clinical conditions not typically linked to Cbl deficiency, including vascular disease ([153](#)), cancer ([154](#)), dementia ([155, 156](#)), Alzheimer disease ([157](#)), birth defects and common pregnancy complications ([158, 159](#)) ([160](#)).

Diagnostic markers of cobalamin function

Serum cobalamin

Serum Cbl comprises the total amount of circulating Cbl, either bound to HC or TC. Measurement of serum Cbl is the most commonly used biochemical test for diagnosing Cbl deficiency, and most patients with clinically confirmed Cbl deficiency have low levels. Concentrations < 150 pmol/l are considered to indicate a Cbl-deficient state. Metabolic tests, like the measurement of plasma MMA and tHcy, have revealed that metabolic signs of Cbl deficiency can occur even though serum Cbl is in the normal range ([161](#)). Moreover, metabolic studies indicate that more than 15 % of patients with low serum Cbl may not have Cbl deficiency, which suggests that a Cbl concentration below the reference interval may be

diagnostically misleading (for review see (162). From these and other observations (for review see (162, 163) it has become evident that serum Cbl has lower diagnostic sensitivity and specificity than plasma concentrations of MMA and tHcy for the detection of mild or sub-clinical Cbl deficiency.

Serum concentrations of total Cbl are dependent on both the amount of available Cbl and on the concentrations of the two Cbl-binding transport proteins. The presence of Cbl or its specific transport proteins in plasma may be influenced by a diversity of factors, including hereditary HC-deficiency (164), ethnic and racial factors (165), haematological disorders (166), the influence of female sex hormones during pregnancy (167) and oral contraception (168, 169). However, serum Cbl concentrations in individuals who are homozygous for the common genetic polymorphism 776C>G in the TC gene are not different compared to controls (48).

Holo-transcobalamin

Less than 30 % of the total amount of Cbl in plasma is bound to TC. Only TC-bound Cbl, denoted holoTC, is available for cellular uptake by receptor-mediated transport, and is considered to be the biologically active fraction of the vitamin (37). HoloTC has a short half-life, varying from minutes to hours, due to rapid cellular uptake (170, 171), and as such may be a parameter responsive to changes in Cbl homeostasis.

The introduction of new methods for the measurement of holoTC (172, 173) have renewed the interest to study the physiologic role of holoTC and to evaluate its usefulness as a marker of Cbl deficiency, where low plasma holoTC (< 37 pmol/L) would indicate a Cbl-deficient state. Supplementation studies performed in non-deficient and Cbl-deficient subjects showed that holoTC was responsive to changes in Cbl supply (174, 175). HoloTC performed as good as or slightly better than serum Cbl itself as a diagnostic marker for the detection of Cbl deficiency (176) (177-180). Further, holoTC turned out to be an early manifestation of a negative Cbl balance (177, 181, 182), and was related to metabolic status, as measured by plasma MMA (183) or tHcy (184, 185).

However, the physiological cycle of holoTC is quite complex, and details of holoTC regulation have not been fully elucidated. Hence, plasma concentrations of holoTC may be affected by several factors, which include not only the amount of absorbed Cbl, tissue requirements of Cbl, but also the rates of hepatic and renal uptake of holoTC, the production and release of ileal (34) and possibly renal (186) holoTC, the common genetic polymorphisms

of the TC gene, TC776C>G ([48]) and TC67A>G, and perhaps other factors related to qualitative and quantitative variations in TC. Several observations point to the kidney to play an important role in holoTC metabolism, as serum holoTC is significantly associated with serum creatinine ([180]), and serum concentrations of holoTC are higher in renal patients compared to controls ([177, 179]). Use of oral contraceptives is associated with changes in Cbl binding proteins ([168, 169]), but it is not known whether the concentration of plasma holoTC is affected.

Methylmalonic acid and total homocysteine

MMA and total Hcy (tHcy), which reflect the activities of Cbl-dependent MCM and MS, are sensitive markers of Cbl status. Therefore, measurement of MMA and tHcy are of clinical value in diagnosis and follow-up of functional Cbl deficiency ([137, 152]).

The metabolism of Hcy and MMA is depicted in Figure 2. Cbl is the only cofactor, which is involved in the degradation of propionyl-CoA via methylmalonyl-CoA to succinyl-CoA. Limited availability of Cbl, apart from enzyme defects, impairs the conversion of methylmalonyl-CoA to succinyl-CoA, and subsequently results in the elevation of MMA. Renal impairment, vascular volume depletion and rare inborn errors affecting MCM are the only other conditions known to cause elevation of MMA ([68, 187, 188]). Therefore, MMA is both a sensitive and a relative specific marker of Cbl function, and plasma concentration of MMA > 0.26 µmol/L are considered to be associated with Cbl deficiency.

Hcy is formed from Met during AdoMet-dependent methylation reactions. It may be salvaged to Met by Cbl-dependent MS, which requires methylTHF as substrate. MethylTHF is derived from 5,10-methyleneTHF by action of vitamin B_2-dependent MTHFR. In the liver and kidneys, Hcy may be remethylated via an alternative reaction, which is catalyzed by betaine-homocysteine methyltransferase, and which uses betaine as methyl donor. Alternatively, it is ultimately degraded through the transsulfuration pathway, which involves the conversion of Hcy to cysteine by action of two vitamin B_6-dependent enzymes (Figure 2) ([189]). Thus, Cbl, folate, vitamin B_6, vitamin B_2 and betaine as cofactors or substrates in Hcy metabolism are determinants of circulating tHcy concentrations ([190-192]). Folate or Cbl deficiency impairs remethylation of Hcy, which is exported from the cells into the extracellular compartment ([193]). Therefore, plasma or serum tHcy is a sensitive marker of both folate and Cbl status ([144]). However, elevated tHcy concentrations are also observed in subjects with unhealthy lifestyles and in pathophysiological conditions, such as renal failure

(see Ref. ([190](#)) and references therein), which renders tHcy a less specific indicator of Cbl function than MMA. In adults, plasma concentrations of tHcy < 15 μmol/L are considered normal.

Modulators of cobalamin function

Nitrous oxide

Nitrous oxide (N_2O) or 'laughing gas' is used as a general anaesthetic, and serves as an analgesic agent for the treatment of pain. It is one of few drugs known to significantly impair Cbl function, and its use in humans is associated with haematological ([194-197](#)) and neurological abnormalities ([198-205](#)) that mimic those seen in Cbl deficiency. Therefore, the gas has been used as an experimental tool to rapidly and efficiently induce Cbl deficiency and to study the biochemical and metabolic changes associated with Cbl deficiency.

Nitrous oxide and methionine synthase

N_2O rapidly inactivates Cbl-dependent MS. This has been extensively demonstrated with purified enzyme ([126](#)), in rodents ([81, 206, 207](#)), in cultured human cells ([128, 208](#)), and in human cells of different origin exposed to N_2O ([209-212](#)). Others have demonstrated an increase in plasma tHcy after N_2O anaesthesia ([213, 214](#)).

Inactivation increases with increasing N_2O partial pressure and with duration of N_2O exposure ([81, 206, 215](#)). Furthermore, N_2O inactivation rates depend on the species and tissue examined ([210, 216](#)). N_2O inactivates MS only if this enzyme is catalytically active, and the inactivation rate is a function of MS activity, as high levels of folate aggravate, and methionine loading alleviates the deleterious effects of N_2O ([128, 143, 193, 213](#)). Therefore, the differences in the susceptibility of MS to inactivation by N_2O in the different tissues may probably be due to different rates of catalytic turnover.

During catalytic turnover, the methyl group of MeCbl bound to MS is transferred to Hcy, which leaves Cbl(I) free to be oxidized by N_2O, resulting in enzyme inactivation. The ability of N_2O to react with transition metal complexes was first described by Banks et al ([217](#)) and further characterized in studies with isolated ([126](#)) and bacterial MS ([218](#)). The oxidation of Cbl(I) forms Cbl(II), N_2 and presumably a potent oxidant that is believed to attack the AdoMet associated with MS, thereby causing conformational changes that block de

novo activation of the enzyme ([218, 219](#)). Some of the enzyme-bound Cbl is released after inactivation and probably modified to Cbl analogues ([81, 218, 220](#)).

The recovery of MS after N_2O exposure is slow and occurs over a period of days. This has been shown in liver of N_2O-exposed animals ([81](#)) and in mononuclear cells from N_2O-exposed patients ([213](#)). Hence, recovery of enzyme activity presumably requires de novo synthesis of the enzyme.

Nitrous oxide and methylmalonyl-CoA mutase

Sparse data exist on the possible effects of N_2O on the function of the other Cbl-dependent enzyme MCM. The excretion of MMA in urine was not increased in rats after brief (< 24 hours) exposures to N_2O ([207](#)). Therefore, the authors considered holoMCM activity to be normal and explained their finding by the fact that enzyme-bound AdoCbl is not directly susceptible to the oxidative effect of N_2O ([207](#)). Prolonged exposure (> 2 weeks) decreased holoMCM activity in rat liver ([81, 130](#)), an effect thought to be secondary to the formation of Cbl analogues and the depletion of the intracellular pool of endogenous Cbl. Total MCM activity was not significantly altered in mononuclear cells from patients subjected to N_2O anaesthesia for 75-230 min when compared to controls ([213](#)). The time course for the recovery of holoMCM activity after N_2O exposure has not been investigated.

Methionine dependency

Met dependency is defined as the inability of a cell to proliferate in a culture medium when Met is replaced by its immediate precursor, Hcy. This metabolic abnormality has been revealed as a common finding in malignant cells of different origin ([221, 222](#)). Most normal cells tested in culture, including fibroblasts and liver, kidney and epithelial cells, are able to grow in a medium devoid of Met, but enriched with Hcy ([223, 224](#)). Cells that proliferate in such medium (Met -, Hcy+) are termed Met-independent cells.

Methionine dependency and cobalamin function

The fundamental biochemical mechanism for Met dependency remains unclear, probably because several biochemical changes create this phenotype. Met dependency linked to Cbl metabolism can result from inactivation of Cbl-dependent MS itself, or from a decrease in the supply of cofactors. Some studies demonstrated low MS activity in Met-dependent tumour cell lines ([223, 225](#)), and defects in intracellular Cbl metabolism leading to lower MS activity

have been described in human melanoma and glioma cell lines (76, 77).

Female sex hormones and serum cobalamin

Female sex hormones may lower serum Cbl values. Reduced serum concentrations have been observed in users of oral contraceptives (OC) (168, 226-228), during pregnancy (167), and in men treated with high doses of ethinyloestradiol for prostate cancer (229). A moderate decrease in serum Cbl was observed in HRT users by some (230, 231), but not all (232, 233) investigators.

Female sex hormones and cobalamin binding proteins

There is evidence from the literature that female sex hormones may affect plasma concentrations of the Cbl-binding proteins, although data are not conclusive. Some investigators reported on higher holoTC in females compared to males (173, 234), whereas others found lower holoTC in women than in men, but only at age $\leq$ 45 years (235). Total TC was not different between genders (236). However, females aged > 50 years have significantly higher total TC than those < 50 years (237). Furthermore, the OC-induced reduction in serum Cbl has been explained by a decrease in Cbl-binding proteins (168). However, data are inconsistent and conflicting (169, 238) (226, 239), probably due to method variability and lack of longitudinal data. Cbl-binding proteins and their respective fractions have not been measured in HRT users, and there are virtually no data for holoTC for either user group exposed to exogenous female sex hormones.

Less inconsistent results have been obtained in studies in pregnant women. During the third trimester, the total Cbl-binding capacity was increased (168, 240), as was apoHC in particular, and to a minor degree apoTC (167), whereas holoHC was decreased (167).

Female sex hormones, methionine synthase and total homocysteine

There is little information on whether and how oestrogens affect the specific activity of MS. Finkelstein and co-workers observed that pharmacological doses of oestrogen caused a significant increase in total MS activity in rat kidney (241). These results may be related to the decrease in plasma tHcy that was observed in another animal study of oestrogen treated male rats (242).

In humans, the effects of both natural and synthetic oestrogens on plasma tHcy concentrations vary according to the formulation and route of administration, with oral

oestrogen therapy being the most extensively studied (243). Plasma tHcy concentrations are lower in premenopausal or pregnant women than in men or in postmenopausal women (244, 245). THcy is higher in the pill-free interval in OC users (246), but OC use in general is not associated with decreased plasma tHcy in females (228, 247-249). However, tHcy levels are significantly reduced in ethinyloestradiol treated men with prostate cancer (229), and both oral and transdermal HRT regimens reduce plasma tHcy concentrations (233, 250-255). The tHcy lowering effect is strongest in women with the highest plasma tHcy concentrations (256, 257) and is not evident in women with low pre-treatment values (230, 258, 259).

Female sex hormones, methylmalonyl-CoA mutase and methylmalonic acid

There are no available data on MCM activity and female sex hormones, and there is no gender difference for the metabolic Cbl marker, MMA (260, 261). However, MMA is significantly lower in elderly women on oestrogen replacement therapy compared to non-users, but an explanation for this finding is not readily apparent (232).

AIMS OF THE STUDY

To assess Cbl function and to study Cbl metabolism during conditions that modulate transport and function of the vitamin

The specific objectives were:

- To construct a fully automated, non-radioactive assay for total and holoMCM activity for use in cell culture and clinical studies,

- to explore the interrelationships of the two Cbl-dependent enzymes and their respective cofactors during Cbl depletion and repletion,

- to further investigate the regulatory network of intracellular Cbl metabolism under various conditions that are assumed to impose a metabolic stress on the function of the vitamin,

- to study Cbl function and distribution under the influence of female sex hormones as supplied by oral contraceptives and hormone replacement therapy.

EXPERIMENTALS AND METHODS

Cell culture studies

Cells and cell culture conditions

The MRC-5 cell line is isolated from normal lung of human male foetus and is referred to as lung fibroblasts. The cells were obtained from the American Type Culture Collection (Rockville, MD). The GaMg human glioma cell line was established in 1984 from a glioblastoma multiforme tumor in a 42-years-old female (262). From this last cell line, a Met-dependent (P60) and a Met-independent (P60H) variant were developed and characterized with respect to intracellular Cbl metabolism (77, 208).

Met-independent P60H cells grow in Met-deplete, Hcy-enriched medium due to their ability to effectively remethylate Hcy to Met. Met-dependent P60 cells are characterized by low catalytic turnover of MS and did not thrive in Hcy medium. Due to their different remethylation ability, the two cell lines served as models to study regulation mechanisms of Cbl metabolism during high and low Cbl-dependent remethylation activity and under various conditions of metabolic strain.

Although the phenotypes seemed stable, parameters may change during many passages and the growth phase, and we routinely used a highly standardized culture procedure. Stem cultures of the cell lines were kept for no longer than 5 passages before a new cell aliquot was thawed, and within identical experimental series, cells were always seeded at the same density in relation to area and volume of culture medium.

All cell lines were cultured as monolayers in Dulbecco's modified Eagle's medium supplemented with glutamine, CN-Cbl, folic acid, Met, and non-essential amino acids in addition to foetal calf serum. Dependent on the experimental design, Met (50 µmol/L) was replaced with Hcy (200 µmol/L) in the culture medium, cells were exposed to N_2O (50 % N_2O, 25 % N_2, 20 % O_2, 5 % CO_2) instead of air (75 % N_2, 20 % O_2, 5 % CO_2), or cells were cultured in the absence or presence of the protein synthesis inhibitor, cycloheximide (3 µg/ml), in the culture medium.

Preparation and storage of rat liver homogenate

A 4-month-old male rat (*Rattus norvegicus*, BD9) was stunned and exsanguinated, and the liver was immediately removed and chilled on ice. All further procedures were carried out at 0-5 ^{o}C. The liver was chopped into small pieces and homogenized in 4.5 volumes (vol./wt.) of 10 mM Hepes buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, using a Potter-Elvehjem homogenizer at 720 rpm and with two strokes of a loose-fitting PTFE pestle. The homogenized tissue was immediately centrifuged at 750 g at 4 ^{o}C for 10 min, and aliquots of the supernatant were stored at −80 ^{o}C. Before assay, liver homogenates were diluted 15-fold in 200 ml/L glycerol and sonicated on ice at 30W for 4 sec. The rat liver was primarily used to develop and validate the MCM assay, and was later used to validate the analytical performance of the enzyme assay.

Cell harvesting, preparation and storage of cell extracts

Human lung fibroblasts and glioma cells grown on culture dishes were brought into suspension by treating with trypsin (0.1 mg/mL) for 10 min at 37 ^{o}C; then the trypsin was inactivated with fresh medium. Cell suspensions were transferred to conical 10 ml tubes, centrifuged at 190 g for 5 min, washed in phosphate-buffered saline and centrifuged again. Cell pellets for the MS assay were frozen undiluted. Cell pellets for the MCM assay were re-suspended in 300 µL 200 ml/L glycerol before storage. Samples for MCM were sonicated on ice at 30W for 4 sec before analysis in order to ensure cell and mitochondrial membrane rupture.

All samples were kept on ice and in red dim light whenever possible in order to protect the Cbl from light inactivation, and they were stored at −80 ^{o}C until analysis.

Preparation of peripheral mononuclear blood cells

Venous blood was drawn into evacuated blood collection tubes containing EDTA, and immediately placed on ice. Mononuclear white blood cells were isolated from whole blood according to the method of Böyum ([263]). The method is based on density gradient centrifugation in a medium containing sodium metrizoate and Ficoll. The isolated fractions included lymphocytes, monocytes, and macrophages. After gently washing with phosphate-buffered saline, the cells were counted in a Coulter counter. The final suspension was centrifuged, and the cell pellets were stored at −80 ^{o}C until analysis.

Clinical studies

Subjects

The study population in paper 4 consisted of female healthcare students and workers (n = 264) older than 18 years recruited from the Haukeland University Hospital, the University School of Medicine and the University School of Social Work in Bergen, Norway.

Ethical approval

The study protocol (paper 4) was approved by the Norwegian Social Science Data Services and the Regional Committee for Medical Research Ethics whose directives are based on the Helsinki Declaration. The participants received oral and written information about the aim of the study, and their right to withdraw from the study, before they gave their written consent to participate.

Data collection

Relevant health information for paper 4 was obtained by a simple questionnaire. On the basis of the type of hormonal preparations used, the subjects were divided into users or controls. Other medication was registered in order to identify subjects on treatment or with diseases known to be associated with changes in Cbl status or metabolic markers. On that basis, five subjects were excluded. All vitamin supplementation was recorded, and subjects were classified as users or non-users of vitamin supplements containing both Cbl and folate. Intake of meat and dairy products was categorized as at least 3 times a week and less often. Smoking habits were analyzed based on the number of cigarettes smoked per day.

Blood sampling and storage

Blood samples (paper 4) were obtained by antecubital venipuncture between 10 am and noon from predominantly non-fasting women. For the preparation of serum, venous blood samples were drawn into blood collection tubes without anticoagulant, and with EDTA as the anticoagulant for the preparation of plasma. The samples used for tHcy determination were placed on ice, and plasma was separated from the blood within two hours. Serum samples were allowed to clot for 30 min before centrifugation. Plasma and serum samples were stored at $-80\ ^{\circ}$C until analysis. The storage time was up to 2.5 years.

Analytical methods

Methylmalonyl-CoA mutase

We used a fully automated non-radioactive method that measures the conversion of methylmalonyl-CoA to succinyl-CoA (264) in rat liver, cultured cells and mononuclear cells. For analysis of total enzyme activity, the enzyme was pre-incubated with AdoCbl, while no exogenous cofactor was added to determine the holoenzyme activity. The assay involved further incubation with the substrate, methylmalonyl-CoA, termination of the reaction by adding trichloroacetic acid, filtration to remove precipitated protein, and finally analysis of the filtrate. The product, succinyl-CoA, was separated from methylmalonyl-CoA using a reversed-phase C_{18} column that was eluted with a methanol gradient. The retention times of methylmalonyl-CoA and succinyl-CoA were 3 min and 4.4 min, respectively. Enzyme activity was expressed as nmol/hour/mg of protein, unless otherwise indicated.

Methionine synthase

In paper 2 and 3, the activity of MS in cell extracts was measured by a slight modification (193) of the radioisotope method developed by Weissbach *et al* (119). In the presence of DL-Hcy, AdoMet, CNCbl, dithioerythritol and mercaptoethanol, Hcy received its methyl group from 5-(^{14}C)methylTHF to render radioactive Met. The radioactive Met was separated on a Bio-Rad AG 1-X8 column, and the radioactivity of the eluate was measured on a scintillation counter. Enzyme activity was expressed as nmol/hour/mg of protein.

In paper 3, the MS activity in cell extracts was additionally measured by two other assays developed by Banerjee and co-workers (104, 109, 116). They also measured the amount of radioactive Met formed by transfer of a methyl group from 5-(^{14}C)methylTHF to Hcy. However, the assays were performed under strict anaerobic conditions, and either titanium citrate or NADPH was used as the source of reducing equivalents. The titanium citrate assay was performed with and without added Cbl, and thus provides an estimate of the percentage of holoenzyme. The NADPH assay is based on the observation that the physiological reducing system can utilize NADPH as the ultimate source of electrons, and offers a mean to activate MS in cell extracts in the presence of endogenous Cbl and the redox-active proteins. Enzyme activity was expressed as pmol/min/mg protein.

Cobalamin reductase

Cell fractionating and analysis of mitochondrial and microsomal CR activity was generally performed according to the method of Pezacka and co-workers ([56](#)). Cultured cells were harvested and homogenized, and mitochondria and microsomes were isolated by differential and density gradient centrifugation. Enzyme markers were used to assess the purity of the fractions. The CR activity was measured spectrophotometrically, following the conversion rate of Cbl(III) to Cbl(II) with NADH as cofactor. Enzyme activity was expressed as nmol/min/mg protein.

Transcobalamin receptor

The assay was performed according to Seligman and Allen ([124](#)). Cultured cells were homogenized in 10 mM Tris-HCL buffer, pH 7.5, containing 140 mM NaCl and 0.1 mM phenyl-methyl-sulfonyl fluoride, and extracted with Triton X-100 (1 %), which completely solubilized the TC-R ([122](#)). Human TC[^{57}Co]Cbl, 2 pmol, was added to the solubilized receptor and applied to DEAE-Sephadex column. The TC-R activity was calculated based on the radioactivity eluted from the column, and was expressed as fmol/10^6 cells.

Intracellular cobalamins

Intracellular Cbls were extracted from the cells by heating in the presence of glacial acetic acid and N-ethyl-maleimide ([77, 265](#)), and the cell debris was removed by centrifugation. All procedures were carried out in red dim light. The supernatant was either used directly for analysis of total Cbl or injected into a reversed-phase column in order to separate the various Cbl forms using a modification ([77](#)) of the chromatographic method described by Jacobsen and Green ([266](#)). Retention times for OHCbl, CNCbl, AdoCbl and MeCbl were 10.5, 12, 14 and 16 min, respectively. The column eluate was collected in 260 µl-fractions, and the concentrations of the Cbl forms in the separate fractions were determined by the radioisotope dilution assay developed by van Kapel and co-workers ([265](#)). Salivary R-binder was used as binding protein. Results were expressed as fmol/10^6 cells.

Cobalamin and folate

Serum Cbl was determined by a *Lactobacillus leichmannii* microbiological assay ([267](#)), and serum and whole blood cell folate were determined by a *Lactobacillus casei* microbiologic assay ([268](#)). The assays were performed in micro titre plates ([269](#)), and adapted to a robotic

workstation (Microlab AT plus 2; Hamilton Bonaduz AG). The linear analytical range was 50-1000 pmol/L for Cbl, and 2-80 nmol/L for folate. Samples for the determination for whole blood cell folate were diluted 8-10 times with sample buffer prior to analysis to fit the linear analytical range. Intra- and interassay variations (CV) for serum folate were 6.0 % and 6.3 %, respectively, for whole blood cell folate 9.5 % and 17 %, respectively, and for serum Cbl 5.4 % and 5.7 %, respectively.

Holo-transcobalamin

The determination of holoTC was based on solid phase capture of TC-bound Cbl, subsequent release of Cbl and analysis by a competitive binding radio-immunoassay (173). The assay has a total imprecision of 12 % at a mean plasma concentration of 14 pmol/L, and 6 % at a mean plasma concentration of 67 pmol/L in singleton analysis. HoloTC was determined in previously unthawed plasma samples that had been stored at $-80\ ^{\circ}$C for up to 2.5 years (paper 4). According to the manufacturer, both plasma (EDTA) and serum specimens may be used with the assay. The reference interval is 37-171 pmol/L.

Methylmalonic acid and total homocysteine

The plasma levels of MMA and tHcy were analyzed by a modification of a gas chromatographic-mass spectrometry method based on ethylchloroformate derivatization (270). At the physiological concentration range, the CVs for the MMA and tHcy assays were 3.2 % and 1.5 %, respectively. The upper reference limit is 0.26 µmol/L and 15.0 µmol/L for MMA and tHcy, respectively.

Creatinine

Serum creatinine was determined non-enzymatically in freshly prepared serum samples by the Jaffe alkaline picrate method for the Hitachi 917 system (F.Hoffmann-La Roche Ltd.). The reference range is 65-100 µmol /L and 75-110 µmol/L for females and males, respectively.

Statistical analysis

Data analysis (Paper 1-3) was performed using mathematical functions given in Microsoft Excel. Other statistical analyses (Paper 4) were performed using the Statistical Package for Social Sciences for Windows NT 4.0 (SPSS version 10.0). R (271) was used for Gaussian-

generalized additive models (GAM) ([272]). Two-tailed P values < 0.05 were considered significant.

Descriptive statistics

Continuous variables from repeated measurements (Papers 1-3) and normal distributed variables (Paper 4) were presented as the arithmetic mean (range) and standard deviation (SD) or standard error of the mean (SEM). Within and between-assay imprecision of the assay procedures (Paper 1), and the procedure imprecision in repeated experiments (Paper 1-3) were characterized by coefficient of variation (CV). Variables showing skewed distribution were ranked in order, and data were given as median (range). The statistical significance of differences of means was assessed by Student's *t*-test, and of medians by Mann-Whitney rank-sum test for comparison between 2 groups. Proportions of categorical data between groups were compared with Fishers' exact test.

Correlation

Determination of the linear regression coefficient (R) was performed for the validation of the automated MCM assay in comparison with the manual assay procedure.

Many of the parameters in Paper 4 were not normally distributed, and data sets were small with outliers. Therefore, we chose a non-parametric correlation method, i.e. Spearman's, to evaluate association between variables.

Regression models

In Paper 4 multiple linear regression models were used to assess the simultaneous relations among the various predictors of holoTC as the continuous variable. Multiple linear regression analyses were also used to examine the influence of OC and HRT on serum Cbl and plasma levels of holoTC, MMA and tHcy, adjusting for other life style factors and predictors.

By means of binary logistic regression analyses, we estimated the ability of OC-use and HRT to independently predict dichotomous outcomes (Paper 4). Skewed dependent variables were log-transformed and results were expressed as odds ratios (OR) with 95 % confidence intervals (CI).

The dose-response relations between Cbl, holoTC, and the metabolites, MMA and tHcy, were studied with a generalized additive Gaussian regression model (GAM) as implemented

in R. The method generates a graphic presentation of the relations between serum Cbl/plasma holoTC and the outcome in hormone users and controls, and allows adjustment for other variables (Paper 4).

RESULTS

Paper 1

Riedel, B., Ueland, P.M., and Svardal, A.M.

Fully automated assay for cobalamin-dependent methylmalonyl-CoA mutase

Clinical Chemistry (1995) 41:1164-1170

We established a fully automated enzyme assay that provided a tool to study total and holoMCM activity in large series of isolated cell or tissue homogenates. The method allowed the unattended analysis of 56 samples per 24 hours.

The central element in the developmental work was to measure stability of reagents, enzyme, and product against the storage conditions needed for automation, to validate the assay under the prevailing assay conditions, and to evaluate the performance of the assay in different cell and tissue homogenates.

MCM enzyme inactivation during storage at 4 ^{o}C was prevented by 200 ml/L glycerol (52.6 ml/L final concentration during enzyme assay) added to the crude cell homogenate. The time course of the enzyme reaction was not affected by glycerol as demonstrated by linearity of product formation for a period of 5 min.

The substrate, methylmalonyl-CoA, was stable under the assay conditions while the product, succinyl-CoA, had decomposed slightly by < 10 % during 20 hours in series of 56 samples. The decomposition product was recovered chromatographically, and the corresponding peak was integrated with the succinyl-CoA peak.

Linearity vs. time was demonstrated for at least 5 min for both total and holoMCM activity, and linearity vs. enzyme concentration up to 13.3 g/L protein and 14.5 g/L protein for rat liver homogenate and cell homogenates, respectively. In addition, the automated method was sufficiently sensitive to determine both holo- and total MCM activity in human lung fibroblasts, glioma cells and rat liver, and had acceptable within-run imprecisions of 2-5 % and 6-9 % for total and holoactivity, respectively. These findings demonstrate that the method was stable for the required time period and for a wide range of protein concentrations, and reliably determined enzyme activity in different cell types.

For validation purpose, the automated method was compared with the manual procedure by analyzing MCM activity in rat liver homogenates containing increasing concentrations of

protein, and a linear regression correlation coefficient of R = 0.99 indicated that the automation process had no negative effects on the enzyme assay.

Additional results

Methylmalonyl-CoA mutase activity in isolated mononuclear cells

published in part in

Christensen, B., Guttormsen, A.B., Schneede, J., Riedel, B., Refsum, H., Svardal, A., and Ueland, P.M.

Preoperative methionine loading enhances restoration of cobalamin-dependent enzyme methionine synthase after nitrous oxide anesthesia

Anesthesiology (1994) 80:1046-1056

The holoMCM activity, i.e. the enzyme activity measured without AdoCbl added to the assay, or the MCM activation ratio, i.e. the activity measured in relation to the total MCM activity, may reflect the functional Cbl status. In order to evaluate the applicability of the MCM assay for the assessment of Cbl status in clinical studies, we attempted to assess both total and holoMCM activity in isolated mononuclear cells from peripheral blood.

Total MCM activity could be detected and measured quantitatively from as little as 0.5×10^6 cells per enzyme assay. In 13 subjects, total MCM activity ranged from 0.91 - 1.73 nmol/min/mg protein, but it was undetectable in two subjects (213). HoloMCM activity could not be measured in the majority of samples. If present, chromatographic peaks of succinyl-CoA were small and often not clearly integrated and distinguished from adjacent peaks.

We concluded that the presented enzyme assay for Cbl-dependent MCM is applicable for the quantitative analysis of total MCM activity in the majority of cases, but not for the assessment of holoMCM activity in peripheral blood cells. Hence, with this method, we could not establish a method based on either holoMCM activity or the activation ratio for MCM as a possible marker of Cbl status for use in clinical studies.

Paper 2

Riedel, B., Fiskerstrand, T., Refsum, H., and Ueland, P.M.

Co-ordinate variations in methylmalonyl-CoA mutase and methionine synthase, and in the cobalamin cofactors in human glioma cells during nitrous oxide exposure and the subsequent recovery phase

In this paper, Met-independent P60H cells were used to investigate the kinetics of both MS and MCM and to measure the levels of their respective cofactors, MeCbl and AdoCbl, during N_2O exposure and a subsequent recovery period of culture in air, with or without the addition of the protein synthesis inhibitor, cycloheximide. This was done in order to gain insight into mechanisms that regulate catalytic MCM and MS activity during Cbl depletion and repletion.

N_2O rapidly inactivated MS, and after a lag period of 6 hours, MeCbl started to decline at a similar rate (0.06/h). After 48 hours of exposure, both MS activity and MeCbl levels were substantially reduced to < 20 %. The reduction of AdoCbl and holoMCM activity was slower (0.04/h vs. 0.02/h, respectively) and the remaining levels after 48 h were about 50 % of those measured in controls. In addition, both OHCbl and total Cbl decreased concomitantly. Notably, holoMCM activity declined at a slightly lower rate compared to its specific cofactor. During inactivation, total MCM activity, including both holo- and apoMCM activity, increased.

During the 24 h recovery period in a N_2O-free atmosphere, MS activity and MeCbl levels increased rapidly. This response was completely inhibited by the protein synthesis inhibitor, cycloheximide. In contrast, the recovery of holoMCM activity and AdoCbl levels was enhanced by cycloheximide.

The changes in AdoCbl during N_2O exposure and the recovery phase closely paralleled the changes in the much larger OHCbl-pool, whereas MeCbl synthesis was dependent on the catalytic turnover of MS.

These findings confirm irreversible damage of MS by N_2O, whereas the restoration of holoMCM activity is dependent on repletion of its cofactor AdoCbl. Levels of MeCbl reflect the catalytic MS activity. The AdoCbl fluctuations induced by N_2O probably reflect variations in the overall intracellular Cbl homeostasis. The close relationship between AdoCbl and OHCbl, and the dependence of holoMCM activity on the availability of its cofactor promoted the idea to explore the holoMCM activity as a marker of intracellular Cbl status.

Paper 3

Fiskerstrand, T., Riedel, B., Ueland, P.M., Seetharam, B., Pezacka, E.H., Gulati, S., Bose, S., Banerjee, R., Berge, R.K., and Refsum, H.

Disruption of a regulatory system involving cobalamin distribution and function in a methionine-dependent human glioma cell line

In order to gain further insight into the mechanisms that regulate the activity of mitochondrial MCM and cytosolic MS, we investigated key processes operating at different levels along the intracellular Cbl pathway in a Met-dependent (P60) vs. a Met-independent (P60H) variant of a human glioma cell line. TC-R activity, microsomal and mitochondrial CR activities, intracellular concentrations of Cbls, and activities of both Cbl-dependent enzymes were measured under standard culture conditions (Met medium), and the results were compared to those obtained under conditions assumed to impose a metabolic stress on the MS pathway (Hcy medium, N_2O exposure).

When cells were cultured in Hcy medium, both cell lines showed an increase in TC-R activity, which was further enhanced by exposure to N_2O. This response was particularly strong in Met-dependent P60 cells, which in contrast to P60H also responded with a 100 % increase in OHCbl and in the microsomal CR activity. However, in P60, there was only marginal (MeCbl) or no (AdoCbl) change in the Cbl cofactors and the specific activities of MS and MCM. In addition, the mitochondrial CR activity was unchanged. Also in Hcy-exposed P60H the mitochondrial CR activity was unchanged, although AdoCbl increased.

N_2O alone (Met medium, N_2O exposure) did not affect TC-R activity in either cell line. However, in P60H, AdoCbl decreased in Met medium, and increased in Hcy medium in response to N_2O exposure. Parallel changes were observed for holoMCM activity. In P60H, MS activity and MeCbl were decreased in parallel during N_2O exposure, irrespective of growth in a Met or Hcy medium.

In conclusion, we demonstrated that low Met and/or high Hcy exert a positive feedback on TC-R activity. The concurrent increase in OHCbl and microsomal CR activity may underlie the same feedback control mechanism, or may be secondary to increased Cbl uptake. This may also be true for AdoCbl and holoMCM activity in Met-independent p60H. The changes in holoMCM activity suggest that the enzyme is indirectly affected by N_2O. Further, the results point to the level of AdoCbl as the factor that regulates holoMCM activity.

Paper 4

Riedel, B., Bjørke Monsen, A.L., Ueland, P.M., and Schneede, J.

Effects of oral contraceptives and hormone replacement therapy on markers of cobalamin status

Clinical Chemistry (2005) 51:778-781

In this study, we investigated the effects of OC or HRT on serum Cbl, and on plasma levels of holoTC, MMA, and tHcy in healthy females.

We showed that OC influence blood concentrations of total Cbl and holoTC. Both parameters were reduced proportionally by about 25 % in women taking OC compared to controls. This reduction was not accompanied by a significant increase of plasma MMA or plasma tHcy. However, in OC users, but not in controls, there was a clear inverse dose-response relationship between plasma MMA and serum Cbl and holoTC, respectively, although MMA concentrations stayed within the normal range. In contrast, in controls, but no in OC users, tHcy showed an inverse relation to serum Cbl and plasma holoTC. These findings may suggest a redistribution of Cbl cofactor between the two Cbl-dependent enzymes, rather than cellular depletion due to OC use. Such hormonal effects may weaken the diagnostic utility of total Cbl and holoTC. Further studies are warranted to decide whether OC users with marginal Cbl status are prone to develop Cbl deficiency.

HRT use decreased the risk of having plasma MMA in the highest quartile, but was not associated with significant changes in circulating Cbl, holoTC or the metabolic markers. These findings, together with the observation of a slightly, though not significantly higher plasma holoTC/Cbl ratio indicate, that HRT does not have a negative effect on Cbl status.

DISCUSSION

Method for methylmalonyl-CoA mutase

We wanted to investigate the activity of Cbl-dependent MCM in samples from large series of experimental and clinical studies, and we aimed to develop a non-radioactive and fully automated enzyme assay coupled to on-line chromatographic separation and detection of succinyl-CoA, based on the assay described by Kikuchi and co-workers (273). For this purpose we used an inexpensive programmable sample processor carrying out complex handling and mixing of liquids combined with automated sample injection into an HPLC column. Such instrumentation had been used previously mainly for automated precolumn derivatization of samples (274, 275), and was shown to confer reliability and high precision to the assays developed in our laboratory (276, 277).

Sample handling and assay conditions

MCM is loosely bound to the inner membrane of the mitochondrial matrix (85), and has to be solubilized prior to enzyme assay. Cell pellets from cultured cells or rat liver homogenates were dissolved in 20 % glycerol, and cell membranes were broken by sonication, as has been done by others (278, 279). Several sonication regimens were tested with respect to power (W) and duration, and with respect to the required sample volume in the appropriate tube. In addition, in order to remove insoluble cell fragments, which might block the needle during the automated assay procedure, we investigated whether use of supernatant after an additional centrifugation would yield a similar enzyme activity compared with the sonicated homogenate. However, the different centrifugation approaches tested with respect to velocity and temperature reduced the formation of succinyl-CoA by more than 50 %. Hence, the samples were not centrifuged, and sonication at 30 W for 4 s in a minimum volume of 300 µl in a conical tube was sufficient to obtain a reproducible enzyme activity.

Enzyme stability during storage is a critical factor. MCM activity was shown to be stable for 18 hours at $-70\ ^{o}C$ in intact rat livers, but not in crude cell homogenates stored at $-20\ ^{o}C$ or at $4\ ^{o}C$ (73). This was in agreement with our own data. For our experiments, enzyme stability was required during storage for up to a week at $-80\ ^{o}C$, during preparation of the cell extracts, and during a maximum pre-assay storage time period of 5.6 hours on the sample rack thermostated at $4\ ^{o}C$. For this purpose, the effect of different concentrations of

antioxidants, including sodium disulfite, mercaptoethanol, dithioerythritol, glutathione and ascorbic acid on enzyme stability were tested. We also tested glycerol, which is known to stabilize enzymes ([280]). Addition of 50 mL/L (final assay concentration 13.15 mL/L) glycerol to the cell extracts yielded the highest MCM activity, and addition of 200 mL/L (final assay concentration 52.6 ml/L) glycerol prevented pre-assay enzyme inactivation for 20 hours. Furthermore, when the cell pellets were resolved in 20 % glycerol before storage at $-80\ ^{o}C$, the total precision of the method improved.

To prevent evaporation of the TCA-treated samples before injection into the HPLC column, we initially covered the assay mixture with bis(3,5,5-trimethylhexyl)phthalate (phthalate), which had been used previously for a similar purpose ([281]). However, phthalate is highly viscous, and occasionally caused either clogging of the needle or reduced volume aspiration, and it was removed from the pipetting needle by a washing procedure with isopropanol instead of water or buffer. With that procedure, a distinct reduction in MCM activity was observed after every 3rd sample due to carry over of isopropanol. We therefore replaced phthalate with a less viscous mineral oil, which, when heated to 37 ^{o}C, was removed easily from the needle by a washing procedure in water, and by the needle piercing a wiping paper.

In conclusion, we showed that the pre-column handling of the cell samples conferred reliability and high precision to the MCM assay.

Chromatographic determination of succinyl-CoA

HPLC is a ubiquitous analytical tool in which the separation of analytes is based on their differential retention by a stationary phase. Different detection systems may be coupled to an HPLC unit. In our system, absorption of succinyl-CoA and methylmalonyl-CoA elution was monitored by UV-detection at 254 nm, and peak areas were quantified by external standardization. The limit of detection for the analytical procedure was determined by measuring progressively more dilute concentrations of cell or tissue samples. Methylmalonyl-CoA and succinyl-CoA were clearly separated, and chromatographic peaks of 0.2 µmol/L succinyl-CoA, corresponding to an enzyme activity of approximately 0.1 nmol/min/mg protein could be quantified. Comparable results were obtained by Kikuchi *et al.* ([273]), and by Gaire *et al.* who used our method with slight modifications ([282]).

Performance of the assay for methylmalonyl-CoA mutase

Our automated device compared favourable with the manual procedure. Kikuchi reported similar MCM activity in cultured fibroblasts when measured by a method based on HPLC or by radioactive detection of succinyl-CoA by paper chromatography (97). Gaire and co-workers showed that the HPLC method was superior to a radio-assay based on the permanganate oxidation of D,L-methylmalonyl-CoA (283) with respect to both sensitivity and reliability. Furthermore, assay precision was comparable with our results (paper 1) (282).

We demonstrated linearity for the enzyme reaction vs. time for at least 5 min, and vs. enzyme concentration for up to 13.3 g/L and 14.5 g/l L for rat liver and glioma cells, respectively. Similar assay characteristics have been obtained by others (282).

The automated enzyme assay was sufficiently sensitive to determine holoMCM activities in rat liver, human fibroblasts and different variants of human glioma cells. Measured total and holoMCM activities in fibroblasts (≈ 1.5 and ≈ 0.25 nmol/min/mg protein) and rat liver (≈ 7 and ≈ 0.3 nmol/min/mg protein) were comparable with published results (99, 100, 273, 279, 282).

In order to evaluate the catalytic MCM activity or the activity ratio (holoactivity vs. total activity) as a parameter to monitor Cbl status in blood samples in clinical studies, we also measured MCM activities in isolated mononuclear blood cells. With our method, we were able to measure total MCM activity in most samples, but not holoMCM activity (213). Also, other authors have failed to measure holoMCM activity with either HPLC (273) or gas chromatography (284). The findings indicate that most MCM in peripheral mononuclear cells exists as apoenzyme, and holoenzyme activities are too low to be measured in this biological material with the presented method.

Method for methionine synthase

In paper 3, MS activity was measured both with the conventional assay (119), and with the titanium citrate and the NADPH assays (104, 109, 116) that are also described in the method section. The holoMS activity was about 100 % of total activity both in P60 and P60H when MS activity was measured with the titanium citrate assay (Paper 3, Table 1). This is in accordance with data of Chen *et al.* who showed that MS is predominantly Cbl-loaded (116), but in conflict with data from Utley *et al.* (105) and Christensen *et al.* (193) who reported that holoMS activity was regularly 10 - 15 % of total activity. HoloMS activity was also different

with the different assays used, i.e. highest with the titanium citrate assay and intermediate (P60H) or low (P60) with the NADPH assay. In addition, with the titanium citrate assay, total MS activity was approximately two- (P60) to four-fold (P60H) higher than with the conventional assay (Paper 3, Table 1).

The mechanisms responsible for the different results obtained with the various methods are not readily apparent. Four variants of Cbl-bound MS can exist, in which the bound Cbl is either MeCbl, Cbl(I), Cbl(II) or Cbl(III). Cbl-bound enzyme containing Cbl(III) and Cbl(II) is inactive, but can be readmitted to the catalytic cycle in a reaction that is independent of added Cbl, but dependent of electron donors (116). The conventional assay, as employed in our laboratory, does not determine MS activity under strict anaerobic condition, and hence, renders the highly reactive Cbl(I) to be susceptible to oxidation. The choice of reductant is crucial for in vitro analysis of MS activity that varied considerably in response to the addition to the assay mixture of two different reductants (mercaptoethanol and dithiothreitol) in variable amounts (75). Also, dithiothreitol has been shown to provide a weaker driving force for Cbl reduction than titanium citrate (116). Weak reducing conditions in the test tube may shorten the delivery of electron equivalents to the enzyme. This leads to an underestimation of enzyme activity, which may explain the low holoMS activity reported previously by Utley *et al.* (105) and Christensen *et al.* (193), and for the low total MS activity obtained with the conventional assay using dithiothreitol compared to data obtained with the titanium citrate assay in paper 3 (Table 1).

With the titanium citrate assay holoMS activity was about 100 % of total both in P60 and P60H cells (Paper 3, Table 1). This agrees with the observation that MS was present predominantly in a holoenzyme form in both porcine liver and human placenta (116), but contrasts with data from Gulati *et al.* who also had employed this assay, and who demonstrated a significant variability in the proportion of holoMS activity in cultured cells ranging from 40-100 % of total activity (285). Moreover, Met-dependent P60 cells had a low intact cell methylation rate (incorporation of radioactivity from [^{14}C]methylTHF into protein) (77), and levels of MeCbl were low (in Hcy medium) or even barely detectable (in Met medium) (Figure 7), indicating low, rather than high catalytic MS activity. The diverging results make it reasonable to ask whether the high proportion of holoMS activity, as measured with the titanium citrate assay, is an artefact. The intracellular presence of significant amounts of trivalent OHCbl and CNCbl (Figure 7) may be a source of error. This Cbl pool can be reduced non-enzymatically by the strong reducing in vitro conditions provided by the titanium

citrate assay, and can effectively favour holoMS formation, as has been demonstrated by Yamada *et al.* (286). Therefore, holoMS activity, as established with the titanium citrate assay, may not measure the functional state of the enzyme, and results obtained in cell extracts should be interpreted with caution.

The NADPH assay even represents another approach for the assessment of holoMS activity, because this assay exploits the presence of the endogenous redox proteins in the cell extracts when the artificial reductants (such as titanium citrate, dithiothreitol and mercatoethanol) are replaced by the physiological reducing agent, NADPH. With this assay, impairment in the reductive activation of MS can be unmasked, as has been shown in fibroblasts belonging to the *CblG* and *CblE* complementation group (285).

The conventional assay allowed us to assess the kinetics of MS inactivation in response to N_2O exposure (Paper 2 and 3), and MS recovery in air with and without protein synthesis inhibition (Paper 2). However, previous results (75, 77, 208, 287), as well as data presented in this thesis (Paper 3) suggest that total MS activity in cell extracts as measured with the conventional assay may not reflect the catalytic turnover in terms of methyl-group transfer from Hcy to Met in intact cells.

In paper 3, both total MS activity and MeCbl declined in the P60 and P60H cells during N_2O exposure, but there was a difference. In P60 cells, total MS activity and MeCbl decreased in a proportionate manner, whereas P60H cells were able to maintain a relatively high level of MeCbl compared to the reduction in total MS activity (Figure 7), and this was most pronounced in Hcy medium (reduction in MeCbl from 57 to 41 fmol/10^6 cells). Furthermore, in Met medium, Met-dependent P60 cells contained only trace amounts of MeCbl whereas total MS activity was rather high. In Hcy medium, the opposite trend was observed (Figure 7). These data are in line with the findings of Fiskerstrand *et al.* (208) who demonstrated that the increase in MeCbl per MS unit was associated with an increase in the intact cell Hcy remethylation rate (incorporation of radioactivity from [^{14}C]methylTHF into protein) despite decreased MS activity (208). They also observed very similar total MS activities in cell extracts of several human glioma cell variants, although their remethylation rates differed considerably (77). Moreover, Hcy export rates, indicating catalytic turnover rates, were not increased despite N_2O induced inactivation of MS in three leukaemic cell lines (193). These results taken together support the view that total MS activity as measured with the conventional assay seems to poorly reflect the functional status of the enzyme.

Regulation of cobalamin-dependent enzymes

The processes that regulate cellular Cbl uptake, cofactor distribution between the two enzymes and formation of the catalytically active MCM and MS have not been not clearly elucidated. The various experimental conditions used in this thesis, such as exposure to N_2O and to cycloheximide, shift from Met to Hcy medium, and in vivo exposure to female sex hormones allowed us to study Cbl function and Cbl distribution in relation to different conditions that are assumed to alter Cbl status. In addition, the use of Met-dependent P60 cells, which in part have biochemical characteristics similar to those observed in *CblC* or *CblD* cells, may provide further insight into regulation mechanisms of intracellular uptake and distribution of the vitamin.

Cofactor formation and enzyme function

MS is regarded to be the primary target of N_2O ([65]). In experimental studies (paper 2 and 3), we observed a time-dependent inactivation of the enzyme activity, as has been consistently observed in vitro and in vivo ([81, 126, 213]). The inactivation of MS activity was accompanied by a temporal and quantitative loss of MeCbl, which is in accordance with data from in vivo studies with rat liver ([81]) and with isolated enzyme from E. coli in vitro ([219]). However, enzyme inactivation preceded the decrease in MeCbl by 6-12 hours in our cell culture experiments (Paper 2). This suggests that oxidative modification of the protein structure is the primary event, which in turn impairs the reductive methylation of Cbl. During recovery in air, MS activity was regained in parallel with MeCbl repletion, and both processes were blocked under conditions of protein synthesis inhibition (Paper 2, Figure 2). Hence, our data demonstrate that MS is covalently modified by N_2O and that de novo synthesis of enzyme is required for recovery of MS activity. Further, MeCbl formation is dependent on catalytic MS activity.

Sparse data exist on the possible effect of N_2O on AdoCbl-dependent MCM. In paper 2, we investigated the changes of the MCM activity associated with the inactivation of MS in P60H cells. We observed that holoMCM activity declined within hours of N_2O exposure at a rate of 0.02/hour ($t_{1/2}$ = 34 hours), and after 48 hours, the residual activity was 65 ± 4 % (Paper 2, Figure 1C). The AdoCbl content declined concomitantly with the holoMCM activity but at a slightly faster rate (0.04/hour, $t_{1/2}$ = 18 hours). After 48 hours, 57 ± 7 % remained (Paper 2, Figure 1D). The reduction of AdoCbl levels and holoMCM activity in response to N_2O exposure is also evident from data obtained in P60H cells in paper 3 (Figure 7, left

panel). The presented data suggest that the decrease of holoMCM activity is related to the reduction in AdoCbl cofactor level and not by irreversible damage of the enzyme, since simultaneous assessment of total MCM activity demonstrated normal activity. The data from the recovery experiment complete this picture: AdoCbl levels and holoMCM activity increased in parallel during inhibition of protein synthesis, which is consistent with recovery from reversible enzyme inhibition.

The fast reduction of AdoCbl levels and holoMCM activity in P60H cells during N_2O exposure is in contrast to data of Kondo *et al.* ([81]). These authors demonstrated rapid loss of MeCbl levels and a proportionate increase of AdoCbl levels in the liver of rats exposed to N_2O, indicating redistribution of available Cbl towards MCM. Alternatively, uptake and metabolism of Cbl was increased due to regulatory feedback signals, such as low Met or high Hcy. Only prolonged exposure (> 2 weeks) resulted in decreased holoMCM activity in rat liver ([81, 130]), which was associated with increased excretion of MMA in the urine, demonstrating that the level of inhibition achieved caused a significant metabolic change in vivo ([130]). This late effect was thought to be secondary to the formation of Cbl analogues and the ultimate depletion of the intracellular pool of endogenous Cbl ([81]). The observed discrepancy may reflect differences in Cbl metabolism of brain cells vs. liver cells ([61]), but may also be related to no apparent retention of Cbl analogues in the P60H cells (Paper 2, Figure 4B), as opposed to the rat liver ([81]).

Several mechanisms for the reduction in cellular AdoCbl content should be considered. Low AdoCbl may be related to inactivation of ATR. However, inhibition of this enzyme is unlikely, since AdoCbl levels actually increased in response to N_2O exposure when P60H cells were cultured in Hcy medium (Figure 7, left panel). The AdoCbl lowering effect of N_2O is probably secondary to reduction of the much larger cellular OHCbl pool. This is supported by the observation (Paper 2, Figures 3 and 4) that the decrease and recovery of AdoCbl closely paralleled changes in OHCbl levels. However, under conditions of high Hcy and low Met (in Hcy medium), AdoCbl levels were rather related to variations in TC-R mediated Cbl uptake, as indicated by the parallel increase in AdoCbl levels and TC-R activity in P60H cells in response to N_2O exposure during culture in Hcy medium (Figure 7, left panel).

In summary, the presented sequence of events that takes place during N_2O exposure points to initial MS inactivation and subsequent Cbl redistribution, which eventually leads to decreased OHCbl and AdoCbl content, and subsequent reduction of holoMCM activity. Under conditions of high Hcy and low Met (culture in Hcy medium), MS inactivation

probably enhances TC-R-mediated Cbl uptake, leading to increased AdoCbl synthesis and holoMCM activity. Thus, holoMCM activity is dependent on AdoCbl synthesis, which in turn is related to variations in the overall intracellular Cbl homeostasis.
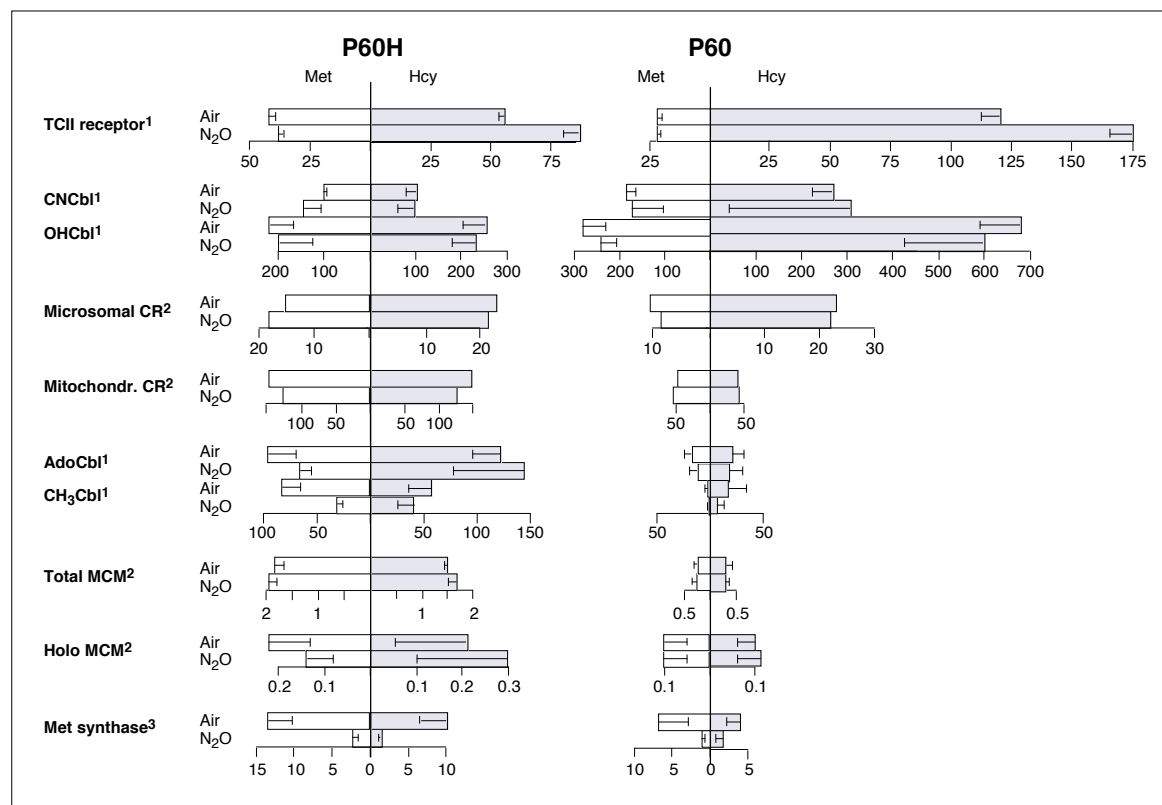
Figure 7. The functional and metabolic profile of the Met-independent P60H (left panel) and the Met-dependent P60 cells (right panel) cultured in either Met (left, open bars) or Hcy medium (right, shaded bars), and exposed to air or N_2O.

The profile is defined in terms of TC-R, cellular Cbls, microsomal and mitochondrial CR, holo and total MCM, and total MS (conventional method). The cells were grown for 48 hours in either Met or Hcy, and exposed to either air (control) or N_2O. The data are mean of 3-4 experiments $\pm$ SD (Cbls, holo and total MCM and MS), 3-4 determinations (TC-R) or 2 determinations (CR). TC-R, transcobalamin receptor; CNCbl, cyanocobalamin, OHCbl, hydroxycobalamin; CR, cobalamin reductase; AdoCbl, adenosylcobalamin; MeCbl, methylcobalamin; MCM, methylmalonyl-CoA mutase; MS, methionine synthase. (Figure presented in paper 3).

[1] $fmol/10^6$ cells

[2] nmol/min/mg protein

[3] nmol/hour/mg protein

Cobalamin distribution

During N_2O exposure, the reduction in MeCbl preceded the slower decline in AdoCbl and the decrease in holoMCM activity (Paper 2, Figure 1). During the subsequent recovery period in air, MeCbl, MS activity, AdoCbl and holoMCM activity increased in this order. However, under conditions of protein synthesis inhibition, the increase in AdoCbl content in particular, and holoMCM activity was actually enhanced (Paper 2, Figures 2C and D). Similarly, during increased TC-R-mediated cellular uptake of Cbl (in Hcy medium) and simultaneous N_2O induced inhibition of MS activity, AdoCbl concentrations and holoMCM activities were increased (Figure 7, left panel). Our observations from both paper 2 and 3 can be explained by low consumption of available Cbl by the MS system, allowing Cbl to be directed towards AdoCbl-dependent MCM.

In paper 4, the observations in OC users compared to controls may provide further evidence for a co-ordinate Cbl distribution between the two Cbl-dependent enzymes. In women not taking OC, the relationship of plasma holoTC and serum Cbl with plasma MMA was weak and non-significant, whereas in OC users, both Cbl fractions showed a strong, inverse correlation with plasma MMA, particularly at low concentrations. Opposite interrelationships according to OC use were seen for plasma tHcy, i.e. strong negative associations were observed in the control group, whereas essentially no relationships were found in OC users (Paper 4, Figure 1). Whether these findings are attributable to the Hcy-lowering effects of female sex hormones, which may antagonize the tHcy increase secondary to impaired Cbl status, or whether female sex hormones may cause intracellular Cbl redistribution favouring supply of Cbl as a cofactor for MS at the expense of the MCM reaction, remains to be elucidated.

Several lines of evidence from the literature support our findings that the MS enzyme may be preferentially supplied with cofactor. Studies with radioactive labelled Cbl showed that in rat liver the MS-Cbl complex was formed at a slightly faster rate than the mitochondrial MCM-Cbl complex (74). Similar results were obtained in studies with normal human fibroblasts grown in a Cbl-supplemented medium, where the formation of OHCbl and MeCbl preceded the synthesis of AdoCbl (55). In addition, inactivation of MS and the associated decrease in MeCbl during short-term exposure (hours) to N_2O was accompanied by a proportionate increase in the amount of AdoCbl in rat liver, suggesting a transfer of Cbl to MCM under conditions of impaired MS activity (81). *CblE* and *G* mutant cells had low MS

activity and low cellular levels of MeCbl, but accumulated more OHCbl and AdoCbl than did control fibroblasts ([83]).

In conclusion, our findings indicate that intracellular Cbl is favourably directed towards MS. The catalytic MS activity seems to play a major signalling role for intracellular Cbl trafficking. The co-ordinated Cbl distribution between cytosolic MS and mitochondrial MCM at a cellular level may account for the variable associations observed between plasma tHcy and MMA and total serum Cbl and plasma holoTC under the influence of female sex hormones.

Regulation of transcobalamin receptor activity

Only sparse data exist on the role of TC-R in regulating Cbl flux across cellular membranes. TC-R protein expression is up-regulated in proliferating lymphoblasts, fibroblasts and hepatocytes ([288]), in leukaemia cells ([289, 290]), and in transplanted sarcomas ([291]). In contrast, upon induction of differentiation, a rapid decline in cell surface binding of holoTC ensued ([289]). TC-R densities on the cell surface correlated inversely with the concentration of Cbl in the culture medium, suggesting that the intracellular content of the vitamin may affect the expression of TC-R ([290]). Further, both cortisone ([292]) and interferon beta ([293]) have been demonstrated to stimulate the expression of TC-R on the cell surface.

There are no data relating the TC-R activity to intracellular Cbl concentrations and the catalytic activity of the two Cbl-dependent enzymes. Hence, we investigated key steps in a sequence of events from binding of Cbl at the cell surface, cellular retention and metabolism, and finally to the activities of the two Cbl-dependent enzymes (Paper 3).

In paper 3, we showed that TC-R activity increased markedly in both P60 and P60H cells after transfer from Met to Hcy medium. This response was further enhanced by exposure to N_2O (Figure 7, left panel). Presumably, these experimental conditions resulted in high cellular concentration of Hcy and low concentration of Met, and the change in either of these amino acids may enhance TC-R activity. Met-dependent P60 cells probably experience the lowest concentration of intracellular Met when cultured in Hcy medium, and these cells had the largest increase in TC-R activity (10 times). Exposure to N_2O in a Met medium, a condition that probably causes high concentrations of both Met and Hcy, did not influence TC-R activity. During N_2O exposure in Met medium, MeCbl and AdoCbl were reduced, but receptor activity was not affected (Figure 7, left panel). Taken together, our findings indicate

that low intracellular Met is a strong metabolic signal for TC-R activity regulation, whereas low levels of cofactors are not. Whether intracellular Hcy, the level of which is probably higher during N_2O exposure in Hcy medium than in Met medium, has a direct role in TC-R activity regulation, is not clarified.

We observed a slight (P60H cells) and a substantial (P60 cells) increase in total Cbl (mainly CNCbl and OHCbl), and a parallel increase in the activity of cytosolic CR after transfer from Met to Hcy medium (Figure 7). These changes may be secondary to the TC-R activation leading to enhanced TC-R mediated Cbl transport across the cell membrane. Alternatively, cellular Cbl metabolism may be directly regulated by product (Met) or substrate (Hcy) of the MS reaction.

Our data provide evidence for a regulatory network involving Cbl uptake, intracellular metabolism, and MS function, and a positive feedback control on TC-R activity by Met depletion may represent a key component of this system. A systematic investigation on the different effects of substrates, cofactor and products of the MS system on TC-R activity and cellular Cbl metabolism was beyond the scope of this thesis, but deserves further attention. Future research may also include investigations whether assessment of TC-R activity levels can be used as a diagnostic marker in Cbl deficiency.

Assessment of cobalamin function

Adenosylcobalamin-dependent methylmalonyl-CoA mutase
The kinetics of Cbl depletion and repletion in paper 2 and 3 were studied under conditions of sufficient extracellular Cbl to restore Cbl function. Thus, provision of AdoCbl to MCM could be investigated in relation to cellular processes involving Cbl uptake, Cbl distribution or metabolism. However, exposure to N_2O may induce conformational changes to MCM, oxidize Cbl(I) which is transiently formed during AdoCbl synthesis, or may convert endogenous Cbl to Cbl analogues, all of which may disturb the interplay between Cbl uptake, distribution, AdoCbl synthesis and finally MCM function.

During N_2O exposure and the subsequent recovery period in air, holoMCM activity decreased and increased in response to changes in AdoCbl levels (Paper 2, Figures 1C and 1D, 2C and 2D), which in turn paralleled variations in the much larger OHCbl pool (Paper 2, Figure 3A and 3D). Notably, recovery of enzyme activity was actually enhanced under

conditions of protein synthesis inhibition, indicating that N_2O does not irreversibly damage the MCM enzyme. Also, total MCM activity slightly increased during N_2O exposure (Paper 2, Figure 1C). Hence, our data demonstrate that the inhibition of holoMCM during N_2O exposure is not related to functional changes of the apoenzyme, but rather to changes in AdoCbl cofactor levels.

In paper 3, the N_2O-induced decrease of holoMCM activity and AdoCbl levels in P60H cells was not accompanied by a parallel decline in OHCbl levels (Figure 7, left panel), as would have been expected from the results in paper 2. An explanation for the observed difference is not readily apparent, but it could be due to slight variations in cell culture or assay conditions. In vivo and in vitro studies with rodent cells have revealed that nitric oxide inhibits holoMCM by reacting with the Cbl(II) or Cbl(I) intermediate in AdoCbl synthesis (99). A similar effect of N_2O on AdoCbl synthesis cannot be ruled out as it may explain the isolated decrease in AdoCbl levels and the subsequent reduction in holoMCM activity. However, such an effect seems unlikely to be of significance in our studies since AdoCbl levels were actually increased during N_2O exposure when the P60H cells were cultured in Hcy medium instead of Met medium (Figure 7, left panel). Moreover, no decline in AdoCbl levels was observed, when MS was completely protected from inactivation by N_2O during simultaneous exposure to methotrexate, a folate antagonist that blocks Hcy remethylation (T. Fiskerstrand, personal communication). These observations suggest that the decrease in AdoCbl levels and holoMCM activity during N_2O exposure is not related to oxidative changes of Cbl intermediates during AdoCbl synthesis.

Transfer from Met to Hcy medium seems to affect Cbl uptake. This is supported by a consistent increase in TC-R activity and concomitant increase in AdoCbl concentrations and holoMCM activity in P60H cells after replacing Met with Hcy. These changes became even more pronounced in the presence of N_2O (Figure 7, left panel, Hcy medium). Increased Cbl uptake in relation to increased TC-R activity has also been shown in Met-dependent P60 cells, although no significant changes in AdoCbl levels were seen, probably due to a defect in cofactor synthesis (Figure 7, right panel, Hcy medium). Interestingly, OHCbl levels did not increase in parallel with TC-R activity and AdoCbl levels. An explanation for this finding is not readily apparent, but it may be due to accelerated flux towards increased AdoCbl synthesis in response to increased TC-R activity.

N_2O has been shown to induce conversion of endogenous Cbl to Cbl analogues, which represented 10 to 40 % of all Cbl species present in rat liver after long-term (weeks) exposure

(81). These analogues are excreted faster than the naturally occurring Cbl forms (81). Synthetic Cbl analogues have been shown to induce Cbl deficiency through inhibition of both MCM and MS in rats (130) and in cultured oligodendrocytes (294). In paper 2, we observed that the cellular Cbl content in P60H cells declined in response to N_2O exposure. (Paper 2, Figure 4B). OHCbl and AdoCbl concentrations decreased to about 50 %, and MeCbl levels declined to < 20 % of control values (Paper 2, Figure 4B). We cannot exclude conversion of endogenous Cbl to Cbl analogues in our system, eventually contributing to the overall decrease in the cellular endogenous Cbl content, further decreasing Cbl-dependent enzyme activities. However, we did not identify by chromatography other Cbl species than the naturally occurring Cbl forms (Paper 2, Figure 4B). Conceivably, P60H cells may rapidly release possible Cbl analogues into the culture medium.

In summary, the concomitant decrease and increase in holoMCM activity associated with alterations in AdoCbl concentrations in response to the variations in OHCbl or TR-R activity provide evidence for the idea that AdoCbl-dependent holoMCM activity in human glioma cells mirrors changes in intracellular Cbl homeostasis. Based on our observations, AdoCbl-dependent MCM may be further investigated as an intracellular marker for the assessment of cellular Cbl status in experimental cell culture studies.

Total cobalamin and holo-transcobalamin

Serum total cobalamin may not reflect intracellular Cbl status in the different tissues and is now considered to have low diagnostic accuracy as an indicator of functional Cbl status (161, 295, 296). It may be strongly influenced by a variety of factors other than Cbl deficiency, including folate deficiency (297), haematological diseases (166), hereditary HC-deficiency (164), ethnic and racial factors (165) and female sex hormones (167, 168, 227-230, 233). In paper 4, we confirmed that female sex hormones, as supplied by OC decreased total serum Cbl concentrations significantly. Moreover, we showed that this decrease was accompanied by a decrease in plasma holoTC. Similar changes were not observed in HRT users compared to controls. Low plasma holoTC in OC users has not been reported previously. The proportional decreases in both Cbl fractions may indicate that Cbl bound to HC and to TC was equally affected in OC users. We have previously shown that total TC is not significantly lower in OC users compared to controls (298), whereas others have found an OC-induced decrease in HC (226, 299). Hence, the mechanism for the observed decrease in plasma holoTC is not readily apparent.

The lower concentrations of total Cbl and holoTC were not accompanied by metabolic signs of Cbl deficiency, as judged by plasma MMA and plasma tHcy concentrations which were within the reference intervals in > 98 % of the OC-users.

In conclusion, HRT does not induce significant changes in the two Cbl markers, whereas OC use causes a decrease in total Cbl and holoTC without metabolic evidence of impaired Cbl status. Such hormonal effects may weaken the diagnostic utility of total Cbl and holoTC.

Total homocysteine

We found no difference in the concentrations of plasma tHcy between women who used OC and those who did not. Moreover, OC-use did not increase the risk of having plasma tHcy in the highest quartile [> 9.2 umol/L; OR (95% CI) 1.2 (0.4-3.8); P = 0.8]. No significant change of plasma tHcy in OC users is in agreement with the literature (228, 247-249). However, in OC users, higher plasma tHcy was reported for the pill-free interval vs. the interval with oestrogen-containing pills in the medication cycle (246), and in the follicular phase vs. the luteal phase of the menstrual cycle (300), which may emphasize a direct Hcy-lowering effect of oestrogens (242), possibly related to an oestrogen-induced increase in MS activity, as has been shown in rat kidney (241). Such an effect may also antagonize a tHcy increase secondary to impaired Cbl function.

In HRT users, we found no significant differences in plasma tHcy levels compared with controls. However, most (231, 253, 255, 256, 301), but not all investigators (230, 258, 259) have demonstrated that postmenopausal HRT actually lowers plasma tHcy. The tHcy lowering effect has been reported to be strongest in women with the highest plasma tHcy concentrations (256, 257) and was not evident in women with low pre-treatment values (230, 258, 259). In our study, median plasma tHcy concentrations were low (< 10 μmol/L) in both user and control groups, which may explain no treatment response for tHcy reported in paper 4.

In conclusion, OC use may reduce the sensitivity of plasma tHcy to identify moderate Cbl deficiency. The mechanisms involved in the variable reported effects of oestrogens as supplied by HRT are not elucidated, and its remains to be investigated whether they are mediated by changes in intracellular folate and Cbl metabolism.

Methylmalonic acid

There are no available data on MCM activity and female sex hormones, and there is no gender difference for the metabolic Cbl marker, MMA (260, 261). In addition, the strong relationship between plasma MMA and serum Cbl that is observed in pregnant women may suggest that female sex hormones do not affect plasma MMA (302, 303). Plasma MMA levels in OC users have not been published before, and earlier studies indicated that urinary MMA concentrations did not differ between OC users and controls (226). No significant effect on plasma MMA in OC users is in agreement with the data from this early study.

In HRT-users, we found no significant differences in plasma MMA compared to controls. However, HRT use lowered the risk of having plasma MMA in the highest quartile [> 0.19 µmol/L; OR (95% CI), 0.31 (0.11-0.93); P = 0.04]. This is in accordance with data from another study investigating postmenopausal women > 60 years (232). The reason for the changes in plasma MMA associated with HRT use is not readily apparent. Whether the trend to lower plasma MMA represents an improvement in Cbl function in elderly HRT users or is unrelated to Cbl status is not evident from our data.

SUMMARY AND CONCLUSIONS

The main purpose of the studies included in this thesis has been to gain further insight into the regulatory mechanisms involved in cellular Cbl uptake, distribution and enzyme function, and to validate the methods used for assessment of Cbl status.

We constructed a fully automated enzyme assay for Cbl-dependent MCM. The method is specific, and has an acceptable degree of precision. The assay allows the unattended analysis of 56 samples per 24 hours. Both holoMCM and total MCM activity could be measured in series of rat liver homogenate and cell extracts from cultured fibroblasts and human glioma cells. Hence, the method proved suitable for use in experimental cell culture studies.

The MCM enzyme assay could be used for the quantitative analysis of total MCM activity, but not for the assessment of holoMCM activity in isolated mononuclear blood cells. Therefore, an activity ratio as a possible marker of Cbl status to be used in clinical studies could not be established.

Formation of cofactors for AdoCbl-dependent MCM and MeCbl-dependent MS is influenced by different regulatory mechanisms. MeCbl formation is dependent on catalytic MS activity, whereas variations in the intracellular amount of AdoCbl seem so be related to overall Cbl content, cellular Cbl uptake as measured with TC-R activity, or to regulatory feed-back signals coupled to MS function.

The changes in holoMCM activity in response to the different experimental culture conditions paralleled the changes in the intracellular amount of AdoCbl. The data emphasize the significance of AdoCbl for catalytic MCM action and support the idea that changes in holoMCM activity reflect changes in overall Cbl homeostasis. Thus, AdoCbl-dependent MCM may be further investigated as an intracellular marker for the assessment of cellular Cbl status in experimental cell culture studies.

The catalytic MS activity seems to play a major signalling role for intracellular Cbl distribution as indicated by the observation that the N_2O-induced loss of Cbl on MS under otherwise Cbl-replete conditions is associated with an increase in AdoCbl levels and

holoMCM activity. Hence, our data support the idea that intracellular Cbl is favourably directed towards MS. The co-ordinated Cbl distribution between cytosolic MS and mitochondrial MCM on a cellular level may account for the variable associations observed between plasma tHcy and MMA and total serum Cbl and plasma holoTC under the influence of female sex hormones as supplied by OC.

Our data provide evidence for a regulatory network involving Cbl uptake, intracellular metabolism, and MS function. A positive feedback control on TC-R expression by Met depletion may represent a key component of this system. Further studies are needed to rule out the differential effects of substrates, cofactor and products involved in Hcy remethylation on the regulation of TC-R activity. Future research may also include investigations whether assessment of TC-R activity levels can be used as a diagnostic marker in Cbl deficiency.

MS and MCM activities may reflect vitamin supply or availability. However, MS activity, as measured with the conventional assay, seems to be poorly related to the functional status of the enzyme, and analysis of MCM or MS activity is not available for use in clinical studies. Measurement of the metabolic markers, MMA and tHcy, and the determination of total Cbl and holoTC in serum or plasma represent established strategies for the assessment of Cbl function. In this thesis, we demonstrate that OC use causes a decrease in total Cbl and holoTC without metabolic evidence of impaired Cbl status. Further, OC use may reduce the sensitivity of plasma tHcy to identify moderate Cbl deficiency. Such hormonal effects may weaken the diagnostic utility of total Cbl, holoTC and tHcy.

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Erratum

On page 24 I wrote: They are classified as being mut^o (enzyme activity not detectable), or mut$^-$ (enzyme activity is detectable) (se Ref. (91) and references therein), but their significance for Cbl function and MCM activity is not clear, and no association with neural tube defects has been found (92).

This shall be replaced by: They are classified as being mut^o (enzyme activity not detectable), or mut$^-$ (enzyme activity is detectable) and are associated with increased concentrations of MMA in plasma and urine (se Ref. (91) and references therein). However, their significance for Cbl uptake and distribution is not fully elucidated, and no association between three MCM single nucleotide polymorphisms and neural tube defects has been found (92).