HETEROGENEITY IN CBLG: DIFFERENTIAL BINDING OF VITAMIN B_{12} TO METHIONINE SYNTHASE

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ABSTRACT

Fibroblasts from patients with functional methionine synthase deficiency can be divided into 2 complementation classes, cblE and cblG. Both have low levels of intracellular methylcobalamin. Both groups also demonstrate low levels of incorporation of label from 5-methyltetrahydrofolate into macromolecules. Under standard reducing conditions, methionine synthase specific activity is normal in cblE fibroblast extracts, but is low in cblG fibroblast extracts. Seven cblE and seven out of ten cblG cell lines demonstrate levels of accumulation of [^{57}Co]CN-Cbl in fibroblasts comparable to that of control cells. They exhibit similar proportions of label associated with the two intracellular cobalamin binders, methylmalonyl-CoA mutase and methionine synthase. The remaining three cblG cell lines exhibit a lower level of cobalamin accumulation, and demonstrate a lack of cobalamin association with the enzyme methionine synthase. The specific activity of methionine synthase is almost undetectable in the three cblG cell lines that showed no such association. These results demonstrate heterogeneity within the cblG group and suggest that the defect in cblG affects the methionine synthase apoenzyme.

SOMMAIRE

Des fibroblastes venant de patients ayant une déficience en synthase de méthionine peuvent être divisés en deux classes complémentaires, cblE et cblG. Les deux démontrent des niveaux réduits de méthylcobalamine intracellulaire. Sous des conditions de réduction standard, l'activité spécifique de la synthase de méthionine est normale dans des extraits de fibroblastes de cblE, et est réduite dans les extraits de fibroblastes de cblG. Sept lignes cellulaires de cblE et sept sur dix lignes de cblG démontrent des niveaux d'accumulation de [^{57}Co]CN-Cbl comparable à l'accumulation dans des cellules controles. En plus, ils ont des proportions comparables de [^{57}Co]CN-Cbl associés avec deux ligands de cobalamine intracellulaire, la mutase de méthylmalonyl-CoA et la synthase de méthionine. Les trois autres lignes cellulaires de cblG démontrent des niveaux réduits d'accumulation de cobalamine, et un manque d'association entre la cobalamine et la synthase de méthionine. L'activité spécifique de la synthase de méthionine est presque non-décelable dans ces trois lignes cellulaires. Ces resultats démontrent de l'heterogeneité dans le groupe cblG et suggèrent que le défaut dans les cblG est au niveau de l'apoenzyme de la synthase de méthionine.

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ABBREVIATIONS

Adenosyl-Cbl	5'deoxyadenosylcobalamin
AdoMet	S-Adenosylmethionine
Ca^{2+}	calcium in the 2+ oxidation state
Cbl	cobalamin, vitamin B_{12}
$Cbl^{1+,2+,3+}$	cobalamin in the 1+, 2+ or 3+ oxidations states
$Co^{1+,2+,3+}$	cobalt in the 1+,2+ or 3+ oxidation states
Btu	B_{12} uptake
CNCbl	cyanoCbl
IF	intrinsic factor
MeTHF	methyltetrahydrofolate
MCM	methylmalonyl-CoA mutase
MMA	methylmalonic aciduria
MS	methionine synthase, methyl transferase
mut	defect in the mutase apoenzyme
OHCbl	hydroxy-Cbl
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMF	proton motive force
TC I, II and III	transcobalamin I, II and III

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

STRUCTURE, MEASUREMENT, AND DISTRIBUTION OF COBALAMIN

1.1 STRUCTURE OF COBALAMIN

Vitamin B_{12}, also known as cobalamin (Fig. 1), is a crucial cofactor for many organisms. Its structure was determined in 1954 by the use of X-ray crystallography in the laboratory of Dorothy Hodgkin (1). Its main skeleton consists of a corrin ring, which is similar to heme, but has cobalt instead of iron as its metal and one less alpha methene bridge than heme. This corrin nucleus is the central structure of all corrinoids, of which cobalamin is a specific group in which the lower axial ligand is 5,6 dimethylbenzimidazole. Three acetamide and four propionamide side chains project above and below the plane of the corrin ring. The lower ligand of this ring is attached to the corrin ring via alfa-D-ribofuranose and the cobalt core. The upper ligand determines the type of cobalamin compound. The upper ligands may be 5'deoxyadenosyl, a methyl group, cyanide, a hydroxyl group, aqua, sulfito and glutathione.

The cobalt core of cobalamin may be found in either of three oxidation states cobalt 3+, cobalt 2+ or cobalt 1+.

1.2 DISTRIBUTION OF COBALAMIN

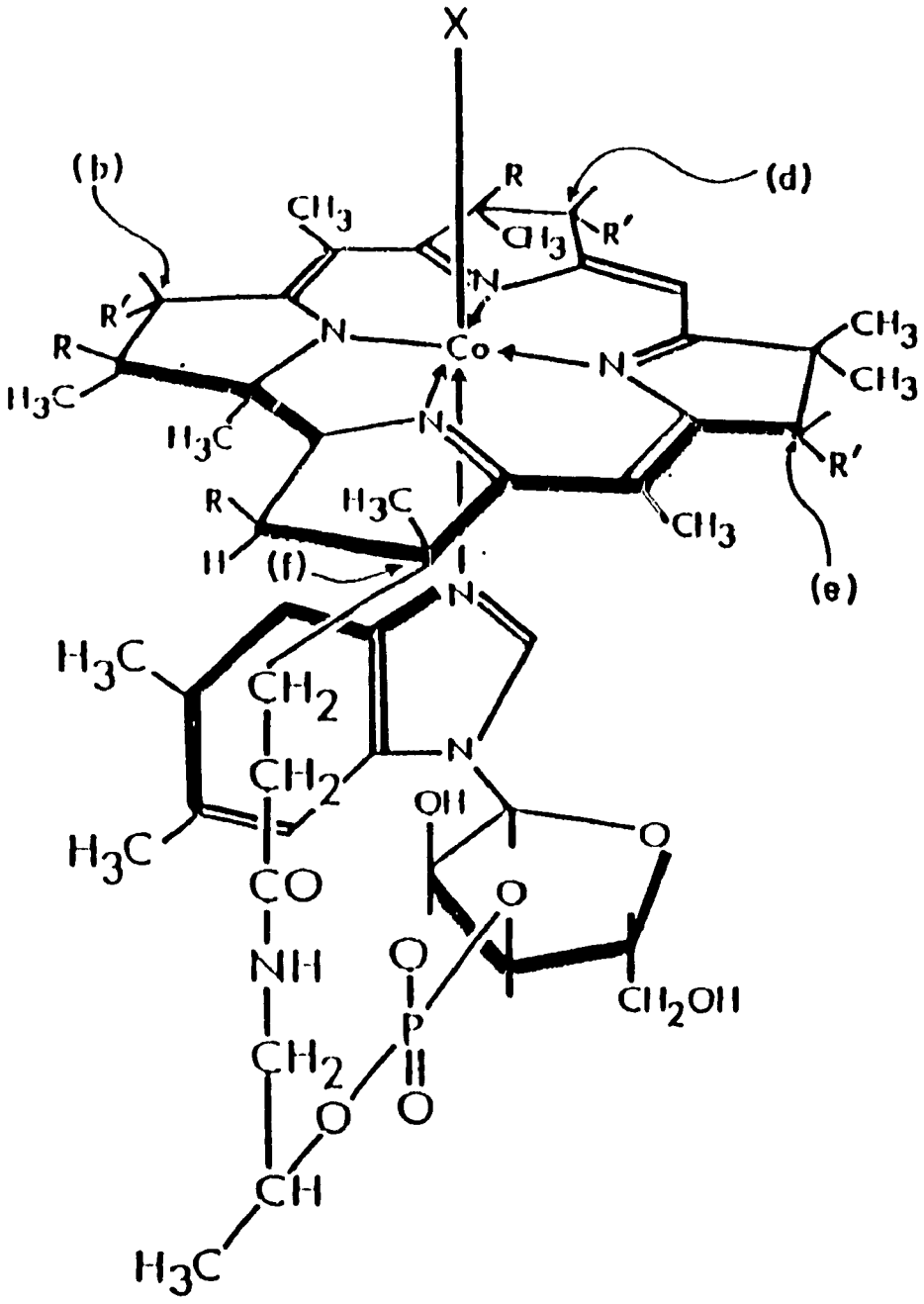
Cobalamin functions as a cofactor for many levels of organisms, ranging from prokaryotes to mammals, although the synthesis of vitamin B_{12} is achieved only by microorganisms (4,5). It is found ubiquitously in animal products and somewhat rarely in plants, but it is all derived from bacteria. Most forms of Cbl (adenosyl, methyl,

aqueous, hydroxy and sulfito) have been found in food; for example adenosyl-Cbl and hydroxy-Cbl are most prevalent in meat, whereas dairy products contain mostly methyl-Cbl and hydroxy-Cbl. Man is wholly dependent on dietary Cbl, even though it is synthesized by the colonic flora (cobalamin cannot be absorbed in the colon) (2,4,5).

1.3 MEASUREMENT AND REQUIREMENTS FOR Cbl

Vitamin B_{12} is measured either by microbiological methods or isotope dilution assays (3,18). The total body content of cobalamin in the adult male is 2-5 mg, and daily estimated dietary requirement is 2-5 μg, so a deficiency state will not arise for years after total cessation of vitamin B_{12} intake. Normal plasma levels can range from 150 to 450 pg/ml which is all bound to transport proteins (the normal range varies among laboratories) (2,3).

FIG.1 THE STRUCTURE OF COBALAMIN R= -CH_2CONH_2; R'= -$CH_2CH_2CONH_2$; X= -CN for cyanocobalamin, -OH for hydroxocobalamin, CH_3 for methylcobalamin, or 5'deoxyadenosyl for 5'deoxyadenosylcobalamin. Reprinted from Fenton and Rosenberg (39).


X
(b)
(d)
(e)
(f)
R
R'
CH3
H3C
N
Co
O
OH
CO
NH
P
CH2
CH
CH2OH

CHAPTER 2

COBALAMIN IN MICRO-ORGANISMS

2.1 COBALAMIN IN MICRO-ORGANISMS

Micro-organisms are a vital component in the vitamin B_{12} pathway, since the de novo synthesis of the corrin ring is restricted to bacteria (6). Micro-organisms are used for the commercial production of Cbl and have also been used for determination of Cbl levels in samples such as human serum (2). The bulk of the work done on bacterial Cbl metabolism has used *Escherichia coli* as the subject, and I will use *E.coli* as the focus of this review.

2.2 Cbl TRANSPORT IN *E.coli*

The *E.coli* membrane is composed of three components, an outer membrane, a periplasmic space and an inner membrane (cytoplasmic membrane). The outer membrane contains porrin channels through which nutrients may enter, but cobalamin is too large for these openings (6). The bacteria has developed a complex and efficient system to retrieve Cbl which usually exists in the environment in low concentrations (usually a range of 10 fM to 1 nM) (8).

Uptake of labelled Cbl by *E.coli* cells is biphasic (6). The initial phase consists of a rapid, energy independent binding to the cell surface. The next phase is a slower, energy dependent transfer into the cell. When *E.coli* comes into contact with cobalamin, uptake is very efficient until internal concentration is as high as 100 μM, within 1/2 generation time. When the medium is exhausted, the intracellular Cbl decreases only by the amount needed for growth (9).

Six components have been identified in the uptake process: proton motive force (pmf), and the products of genes BtuB, BtuC, BtuD, BtuE and TonB (these genes have been mapped (16,10,17,12)).

2.2.1 The BtuB protein

The binding site of the outer membrane for Cbl is a 60 kDa protein, and is called the BtuB protein (or the BtuB gene product) (8). This protein has a high affinity for Cbl, and has a dissociation constant of 0.1 to 5 nM. It is a multivalent receptor, and is responsible for not only Cbl uptake, but it also competitively binds bacteriophage BF23 and the BtuB group colicins (this activity is restricted to only newly synthesized receptor proteins) (6). BtuB exists at roughly 200-300 copies/cell in cells that are grown with no Cbl in the media. If grown in Cbl, there is an inhibition of receptor activity; growth in presence of 100nM of Cbl reduced uptake by as much as 90% (11). Studies have shown that the regulation seems to take place at the translational level with the synthesis of BtuB being repressed by the presence of Cbl (28).

2.2.2 TonB and PMF

The proton motive force, TonB gene product and a second site on the Cbl receptor (in addition to the Cbl binding site) are required for Cbl transport across the outer membrane (15). Together, they interact with the Cbl receptor to lower its affinity for Cbl, thus releasing it into the periplasmic space (6,8). It was observed that 2,4 dinitrophenol inhibited internalization of Cbl: this suggested that Cbl transport into the periplasm was dependent on PMF (6). The TonB protein was observed to be tethered to the inner membrane, but was functionally inside the periplasmic space. It was also

observed that a low pH and a low Ca^{2+} concentration in the periplasmic space lowered the affinity of the Cbl receptor for its substrate. From these observations it was suggested that the TonB protein functions by restricting Ca^{2+} binding on the periplasmic side of the receptor (8). Cellular localization studies suggest that TonB is in fact periplasmic (12). TonB also functions in the transport of ferric iron, bacteriophage T1, phage 80 infections, and several colicins (6).

2.2.3 BtuC, BtuD and BtuE

The BtuC and BtuD gene products are necessary for the transport of Cbl across the cytoplasmic membrane, and fractionation studies suggest that they are membrane associated (17). A 22 kDa protein which had high affinity for Cbl was observed in *E.coli* cells and fractionation studies suggest that this protein is periplasmic (8,17). More recently, a 22kDa protein was found to be the product of the BtuE gene and resided in the periplasm. Although it was not demonstrated that the BtuE protein bound Cbl, this protein in all likelihood the Cbl carrier identified in the aforementioned study (8,10,17).

2.3 *E.coli* TRANSPORT MUTANTS

Mutants have been identified at every gene locus involved in vitamin B_{12} metabolism in *E.coli*, and have been invaluable in the investigation of the pathway.

The mutant in the receptor function is designated the btuB mutant, and is either deficient in energy-dependent transport across the outer membrane (class I mutants), or else lacks binding ability for Cbl (class II mutants) (16). TonB mutants also are unable to internalize Cbl due to their inability to stimulate the release of Cbl from the receptor (12). BtuC and btuD mutants fail to transport Cbl across the inner membrane (14,15).

BtuE, however, does not seem essential for Cbl transport (10).

CHAPTER 3

COBALAMIN TRANSPORT IN MAMMALS

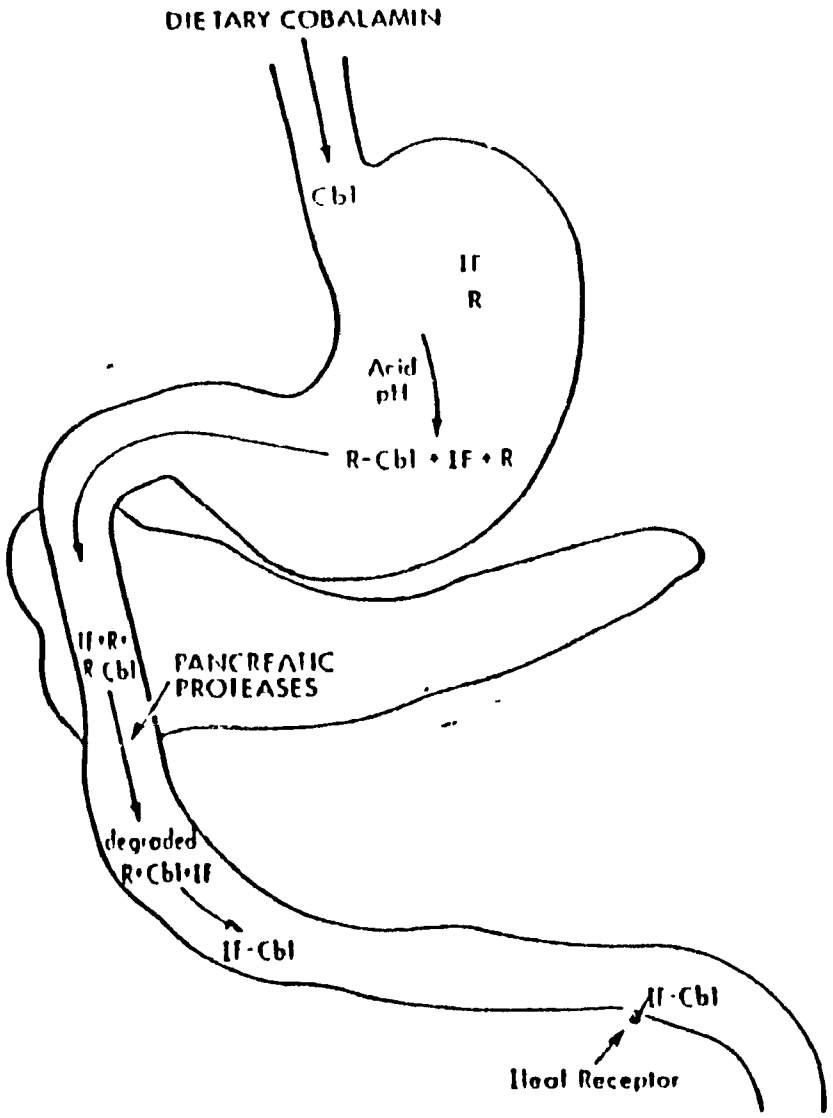
3.1 Cbl TRANSPORT IN MAMMALS

Dietary Cbl is mostly bound to protein at the time of ingestion, and is subsequently released by the action of acid and pepsin in the stomach. Cbl is then bound by a group of proteins called **R binders**, named such for their rapid motility during electrophoresis (20). R proteins are glycoproteins which have a high affinity for Cbl, and are present in plasma, most secretions, and in the cytoplasm of many cells (eg. platelets, erythrocytes). TCI, TCIII, cobalophyllin, and haptocorrin are examples of R binders (20,22,23). They have a high affinity for many corrins, and while their physiological significance is still unclear, it has been suggested that they play a role in the removal of potentially harmful Cbl analogues from the circulation (21,23). R binders in the plasma are cleared by the liver, and then released into the bile. Here Cbl may be more specifically reabsorbed in the small intestine. R binders in the gut may absorb Cbl analogues in the food, and may also play a bacteriostatic effect by denying availability of Cbl and Cbl analogues to bacteria which may require them for growth (20).

3.2 INTRINSIC FACTOR

Once Cbl has been digested free of R binders in the small intestine by pancreatic enzymes, it then binds to **intrinsic factor** (21,23). This protein is synthesized in the

FIG.2 **PROPOSED SCHEME FOR THE ABSORPTION OF DIETARY COBALAMIN.** IF = Intrinsic factor; R = R binder. Reprinted from Seetharam and Alpers (20).


DIETARY COBALAMIN
Cbl
IF
R
Acid
pH
R-Cbl + IF + R
IF•R•
R Cbl
PANCREATIC
PROTEASES
degraded
R•Cbl•IF
IF-Cbl
IF-Cbl
Ileal Receptor

fundic mucosa of the stomach, more specifically, the parietal cell. Its production and release is stimulated by hormones and food. IF recognizes a more narrow range of corrins, and where R proteins bind Cbl more tightly, IF binds Cbl more stringently (22,23). The Cbl-IF complex binds to specific receptors present on the luminal side of enterocytes located in the brush border of the distal ileum. This binding requires the presence of Ca^{2+} ions and a pH between 5 and 7.5 (23). After the attachment of the Cbl-IF complex to the receptor, there is a delay of 3 to 4 hours before Cbl appears in the portal circulation (19). The complex is taken up into the enterocyte through an energy dependent mechanism (23) and Cbl is released from IF in the enterocyte. The mechanism of this release is still uncertain but has been shown to occur at a pH of 5, which is consistent with localization to lysosomes or endosomes (19). There has not as yet been data to show recycling of IF to the brush border membrane.

3.3 TRANSCOBALAMIN II

It is in the enterocyte where Cbl binds to the protein carrier TC II. This protein has a putative molecular weight of 38,000, and is present in plasma, spinal fluid, semen and extracellular fluid (20,22). TC II is made by a variety of cells, including fibroblasts, endothelial cells, amniocytes, and some others (not in lymphoblasts) (22). TC II accounts for only a small amount of total Cbl binding activity in human plasma, but it represents the only phsiologically active Cbl available (20). All newly absorbed Cbl will be found bound to TC II in the circulation.

3.4 Cbl ENTRY INTO CELLS

The TC II-Cbl complex binds to specific receptors which are thought to be present in all human cells (23). Binding requires the presence of calcium ions, and then is followed by an energy dependent endocytic internalization. A very small amount of TC II-Cbl has also shown to have been taken up by nonspecific pinocytosis (26).

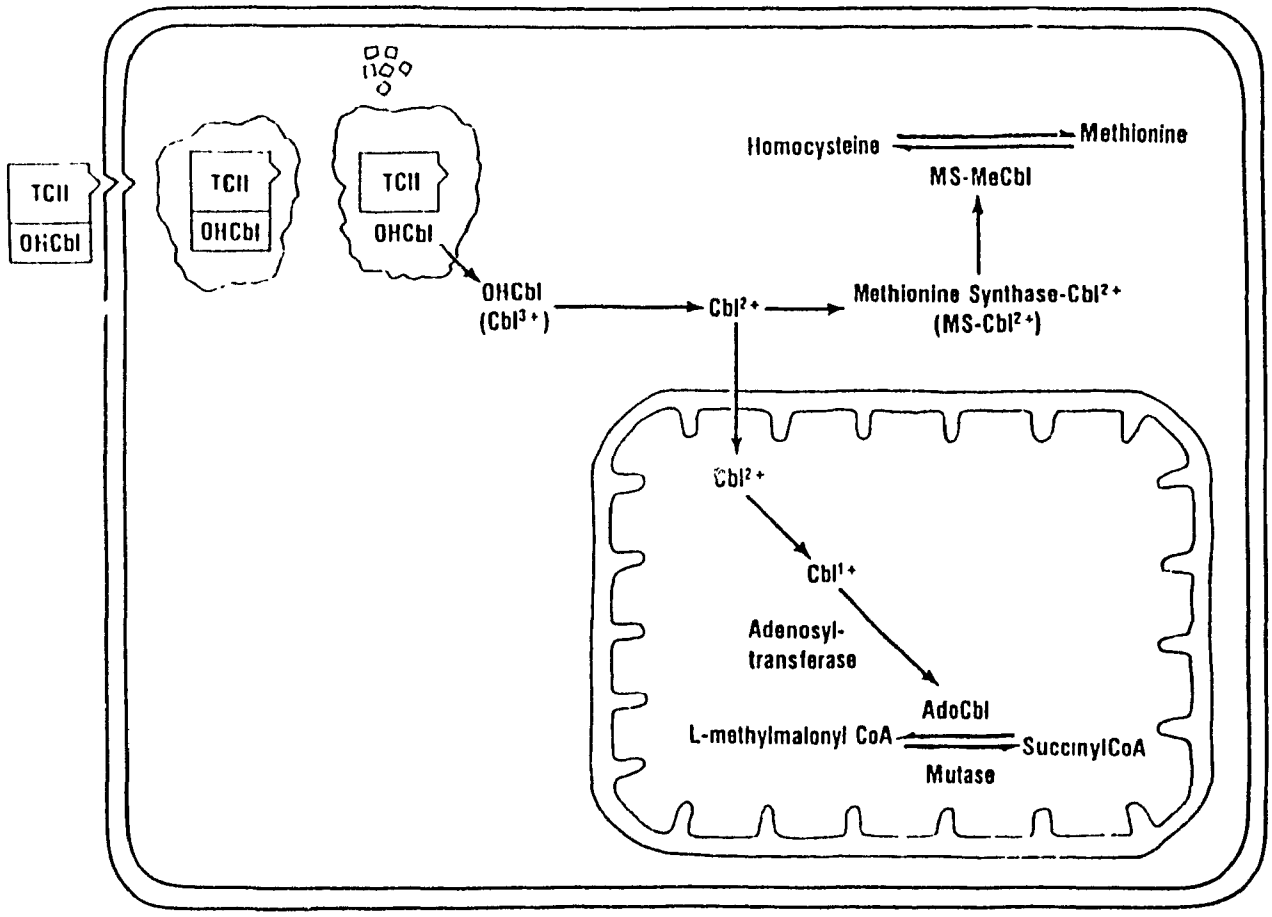
Evidence that cells have the ability to take up free Cbl has also been uncovered. This process contains a specific, saturable, low capacity component which is Ca^{2+} independent, and requires an intact electron transport chain (108). This process could explain the improvement of patients with TC II deficiency that are treated with high doses of OHCbl or CNCbl.

3.5 INTRACELLULAR Cbl METABOLISM

Once the TC II-Cbl complex is internalized, Cbl is digested free in the resultant lysosome, and TC II leaves the cell in fragments (26). The release and digestion of the complex and subsequent efflux of Cbl into the cytoplasm has been shown to be inhibited by chloroquine, which accumulates in endosomes and prevents the generation of a low pH. This demonstrates the need for a low pH to generate the release of Cbl from TC II (22). At this point, Cbl may now efflux across the endosomal membrane into the cytoplasm. As of yet, the mechanism of the efflux is not known. Once in the cytoplasm, the cobalt core of Cbl may undergo a reduction from Co^{3+} to Co^{2+}, and Cbl^{2+} may then bind to methionine synthase and become methylated to form methyl-Cbl. At this point it is ready to participate in the methylation of homocysteine to form methionine. The other option Cbl has in the cytoplasm is to move into the mitochondrion in the Cbl^{3+} or Cbl^{2+} form. Once in the mitochondrion Cbl is further reduced to Cbl^{1+}, and is then

converted to adenosyl-Cbl. At this point, Cbl is ready to assist methylmalonyl-CoA mutase in the isomerization of methylmalonyl-CoA to succinyl-CoA.

FIG.3 **GENERAL PATHWAY OF INTRACELLULAR COBALAMIN METABOLISM.** TCII = transcobalamin II; OHCbl = hydroxocobalamin; $Cbl^{1+,2+,3+}$ = cobalamin with 1+, 2+, or 3+ oxidation state of its cobalt core; MS-MeCbl = methionine synthase bound methylcobalamin; AdoCbl = 5'deoxyadenosylcobalamin. Reprinted from Cooper and Rosenblatt (22).


TCII
OHCbl
TCII
OHCbl
TCII
OHCbl
OHCbl
(Cbl3+)
Cbl2+
Homocysteine
Methionine
MS-MeCbl
Methionine Synthase-Cbl2+
(MS-Cbl2+)
Cbl2+
Cbl1+
Adenosyl-
transferase
AdoCbl
L-methylmalonyl CoA
SuccinylCoA
Mutase

CHAPTER 4

COBALAMIN-DEPENDENT ENZYMES

4.1 COBALAMIN-DEPENDENT ENZYMES IN MICRO-ORGANISMS

The most extensive work done on micro-organisms has focused on *Escherichia coli*, therefore this is the only organism I will discuss.

Cells of *E.coli* cannot make the corrin ring but they have the enzymes to convert CNCbl or AquoCbl into coenzyme forms. There are two Cbl-dependent enzymes in *E.coli*: the adenosyl-Cbl-dependent **ethanolamine-ammonia lyase** (EC 4.3.1.7), and the methyl-Cbl-dependent **methylfolate-homocysteine methyltransferase** (EC 2.1.1.13). The first enzyme is required only when ethanolamine is the sole nitrogen source available The Cbl-dependent methyltransferase, which is the product of the **MetH** gene, is responsible for the biosynthesis of methionine. *E.coli* also possess a Cbl-independent methyltransferase which is the product of the **MetE** gene (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, EC 2.1.1.14), and thus they can grow in the absence of methionine and Cbl. However, *E.coli* cells do prefer to use the Cbl-dependent enzyme if Cbl is available. In fact, the MetH holoenzyme represses the MetE gene product (6).

The MetH gene product is a monomeric enzyme in *E.coli* B, and contains 1 mole of cobalamin per mole of enzyme. It also contains 1 mole of copper per mole of enzyme-bound cobalamin (in addition to cobalt), but since the strain of *E.coli* named K-12 lacks stoichiometric copper (0.1 mol/mol Cbl), it probably does not play a crucial catalytic role (30).

Tryptic digestion of the native recombinant enzyme from *E.coli* yielded 2

fragments of 95 and 35 kDa on a nondenaturing gel which resulted in a loss of activity (106). Further digestion resulted in the instability of the 95 kDa fragment and the subsequent appearance of a 68 kDa and a 28 kDa fragment. On the nondenaturing gel a 28 kDa fragment was associated with Cbl (it was pink on the gel, and methyl-Cbl is pink in colour), and is thus believed to encompass the Cbl-binding region of the enzyme. From sequence analysis, the secondary structure of the Cbl-binding domain is believed to be comprised of alternating alpha-helices and beta-sheets (106).

The MetH gene product uses the mono and polyglutamate forms of 5-methyltetrahydrofolate as methyl group donors to the Cbl to form methyl-Cbl (the metE gene product uses only polyglutamate forms). However, the enzyme must be activated for catalysis by reductive methylation involving S-adenosylmethionine and an electron donor (30). Studies have uncovered the reducing system used by *E.coli*, and it involves two components called the **R component** and the **F component**. The R component is an NADPH-dependent flavoprotein which contains FAD as its prosthetic group, and the F component is a flavodoxin which has FMN as its prosthetic group (31). The electron transfer sequence seems to be NADPH to R to F to methionine synthase (the M component). The activation of the **M component** (MetM gene product) is presumed to involve the reduction of enzyme bound Cbl from cob(II)alamin to cob(I)alamin, and then the subsequent methylation of cob(I)alamin by AdoMet to form MeCbl (31,32).

The mechanism of this reduction in vivo has previously been puzzling since the low midpoint potential (-526 mV for the cob(II)alamin/cob(I)alamin couple bound to MS at pH7) of this redox couple renders this reaction thermodynamically unfavourable. It has been determined that the highly exergonic reaction of the methyl group transfer from AdoMet to cob(I)alamin could be used to drive the endergonic reduction of cob(II)alamin

at potentials as high as -82 mV. This explains the need for AdoMet as a methyl group donor for activation of the enzyme, since the methyl group transfer from MeTHF does not release enough energy to drive the initial reduction (the redox couple potential in the presence of MeTHF rises only to -450 mV)(7).

The MetH gene has been cloned (33), using methionine-requiring nutritional auxotrophs of *E.coli* that were deficient in the MetH or MetE gene products, metH$^-$ and metE$^-$. MetH and MetE genes have been shown to be subject to positive regulation by the **MetR** gene product which is a trans-acting transcriptional activator, and binds to an upstream region in the *E.coli* MetE gene. A similar sequence has been discovered upstream of the MetH gene. AdoMet has also been demonstrated to act as a major regulator of methionine biosynthesis by combining with an aporepressor protein, the **MetJ** gene product, to inhibit transcription of enzymes involved in methionine biosynthesis (30).

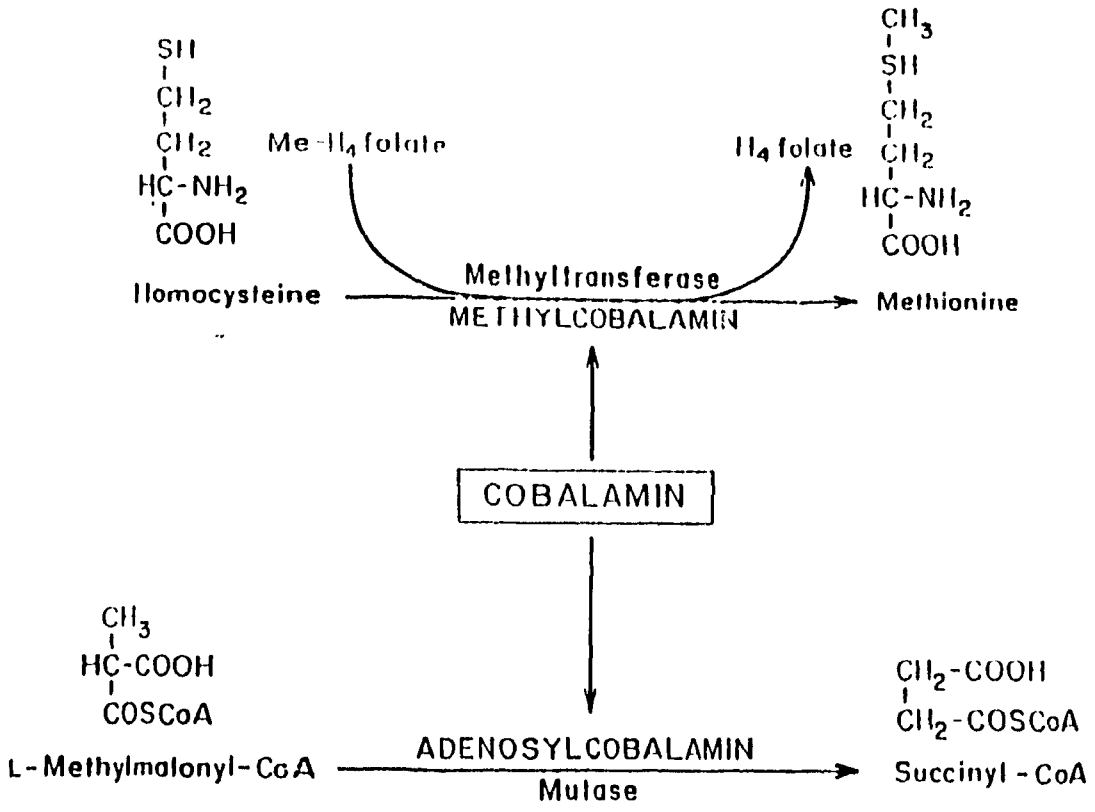
4.2 COBALAMIN-DEPENDENT ENZYMES IN MAMMALS

4.2.1 Methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase (MCM) (EC 5.4.99.2), is a mitochondrial enzyme which uses 5'adenosyl-Cbl as a cofactor and catalyzes the isomerization of methylmalonyl-CoA to succinyl CoA, although this pathway is not a quantitatively important source of succinate in mammalian cells. This is an intermediate step in the degradation of valine, isoleucine, threonine, methionine, odd chain fatty acids, cholesterol, and thymine (34).

MCM is found in most mammalian tissues, and the holoenzyme has been found to have a molecular weight of 165,000, with 2 molecules of adenosyl-Cbl bound per molecule of enzyme (35).

FIG.4 COBALAMIN-DEPENDENT INTRACELLULAR REACTIONS. Reprinted from Fenton and Rosenberg (67).


SH
CH2
CH2
HC-NH2
COOH
Homocysteine
Me-H4 folate
H4 folate
Methyltransferase
METHYLCOBALAMIN
CH3
SH
CH2
CH2
HC-NH2
COOH
Methionine
COBALAMIN
CH3
HC-COOH
COSCoA
L-Methylmalonyl-CoA
ADENOSYLCOBALAMIN
Mutase
CH2-COOH
CH2-COSCoA
Succinyl-CoA

MCM has recently been cloned and sequenced (36) and located to chromosome 6p12-21.2. The enzyme is a homodimer with identical subunits of 72-77x10^6 Da (37). This mitochondrial protein is encoded by a nuclear gene and is synthesized in the cytoplasm as a larger precursor. The protein then undergoes transportation to the mitochondrial matrix where it is post-translationally processed; these two events are tightly linked and are energy dependent (38).

4.2.2 Adenosyl-Cobalamin

Adenosyl-Cbl is the form in which Cbl acts as a cofactor for the mutase enzyme, and is synthesized in the mitochondrial matrix by the enzyme 5'-adenosyltransferase (EC 2.5.1.17). Upon entry into the mitochondrion, cob(III)alamin or cob(II)alamin undergo a reduction to cob(I)alamin, which is then converted to 5'deoxyadenosyl-Cbl by the enzyme adenosyltransferase, a mitochondrial matrix enzyme. It was shown in 1978 (39) that intact rat liver mitochondria were capable of making adenosyl-Cbl from OHCbl, requiring only a source of reducing equivalents (glutamate or NADH) and a source of ATP. This confirmed previous observations that combined cob(II)alamin reductase-adenosyltransferase activities are present in human skin fibroblasts. This also suggests the presence of cob(III)alamin reductase. To date, no transport system has been demonstrated for the entry of Cbl into mitochondria.

4.3 METHIONINE SYNTHASE

Methionine synthase (5-methyltetrahydrofolate:homocysteine methyltransferase) (EC 2.1.1.13) is a cytosolic enzyme which catalyzes the methylation of homocysteine to form methionine. Methyl-Cbl is the cofactor form of Cbl for this enzyme. Methionine

is an essential aminoacid in mammals, and is used in protein and in AdoMet synthesis. The mammalian enzyme has not been as well characterized as the metH gene product, but both appear very similar in their catalytic properties.

MS has not yet been cloned, but it has been isolated from several animal sources including porcine kidney and liver (41,42) and from human placenta (40). MS has been localized to chromosome 1 through the search of MS activity in rodent-human cell hybrids from which human chromosomes were lost with each passage (43). MS purified from human placenta was demonstrated to have a molecular weight of 160,000, and appeared to be a monomer. On an unreduced polyacrylamide gel, human placental extract yielded a major band of 160,000, and only traces of two other bands of 80,000 and 70,000 On a reduced gel, human placental extract yielded three bands of 90,000, 45,000, and 35,000, which suggests a somewhat complex subunit structure. MS contains 2 moles of iron per mole of enzyme, which may be related to a reducing function. It has been postulated that the lower subunits may bind the iron and act as FAD and FMN as in the micro-organismal system (see section 4.1) (40).

The specific activity obtained for the enzyme isolated from human placenta was 0.014 μmol min^{-1} mg $prot.^{-1}$ at 37^0 C (40). This corresponds to a turnover number of 2.2 min^{-1}. The pig liver enzyme, however, had an estimated turnover number of 670 min^{-1} (42), and the partially purified pig kidney MS demonstrated a specific activity of 0.9 umol min^{-1} mg $prot.^{-1}$ (41). These results suggested that the enzyme isolated from the human placenta may have been grossly inactive (30). The turnover obtained from the pig liver MS corresponded more closely to that obtained for the E.coli enzyme, 1300 min^{-1} at 37^0 C (107).

MS activity has been demonstrated in human fibroblasts, and maximal activity was

observed in late stationary phase fibroblast cells (111). In most other cells studied (eg. human lymphoblasts), however, MS activity has usually been highest during the rapid growth phase (44). Although MS is close in size to MCM, the two have been successfully separated on a native polyacrylamide gel (62). MS is present in most tissues, but not in the mucosa of the small intestine (44).

4.3.1 Methyl-Cobalamin

Methyl-Cbl is the coenzyme form of Cbl for the formation of methionine from homocysteine, a reaction catalyzed by the enzyme methionine synthase. Methyl-Cbl is the major circulating Cbl species, accounting for 60 to 80% of total plasma Cbl (52). The formation of methyl-Cbl occurs once cob(II)alamin has bound to MS and receives a methyl group from either AdoMet or 5-MeTHF (see section 4.3.2).

4.3.2 S-Adenosylmethionine

Adenosylmethionine (AdoMet) is a crucial methyl group donor involved in the biosynthesis of a variety of compounds, such as epinephrine and creatine.

MS requires AdoMet and a reducing system to activate the catalytic activity of the enzyme, as in the case of the *E.coli* methyltransferase. AdoMet provides the initial methyl group for the methylation of cob(II)alamin bound to MS (45). After the methylation of homocysteine, cob(I)alamin is left bound to the enzyme. Subsequent methyl groups are then provided by 5-methyltetrahydrofolate, until Cbl undergoes a spontaneous oxidation to cob(II)alamin, whereupon it once more requires AdoMet and a reducing system to generate methyl-Cbl. In stopped-flow kinetic studies, evidence was obtained that both cob(I)alamin and methyl-Cbl are kinetically competent catalytic

intermediates (30).

AdoMet may be required for activation in order to drive the highly endergonic reduction of cob(II)alamin (7), as described in section 5.1. AdoMet also is involved in the regulation of methionine synthesis, with high levels inhibiting the production of methionine (46).

An MS-associated reducing system has not yet been demonstrated in mammals in vivo, but partially purified enzyme is associated with thiol oxidase activity, and thus thiols may serve as the physiological reductants for the mammalian enzyme (47).

4.3.3 5-Methyltetrahydrofolate

Conservation of cellular methionine is only one function of MS. Another important function is the regeneration of tetrahydrofolate, which is essential in other pathways of one carbon metabolism, including the biosynthesis of purines, pyrimidines, and serine. The enzyme methylenetetrahydrofolate reductase (E.C. 1.5.1.20) catalyzes the reduction of CH_2THF to MeTHF, a reaction that is irreversible, and commits folate-bound 1-carbon units for the methylation of homocysteine. MeTHF is the major circulating form of folate, and if MS is nonfunctional, 5-MeTHF cannot be converted to THF, which is needed to replenish the cellular folylpolyglutamates. The result is a depletion of CH_2THF which is required for thymidylate synthesis, and an accumulation of 5-MeTHF which is called the cellular **methyl trap hypothesis**. The total intracellular folate then decreases, due to the fact that 5-MeTHF is a poor substrate for the enzyme folylpolyglutamate synthase, which generates folylpolyglutamates (folate compounds with more than one glutamic residue attached). These forms are essential for the intracellular retention of the vitamin, since the monoglutamate forms may diffuse out of the cell (30).

Methionine synthase uses MeTHF in the polyglutamyl form only slightly preferentially over the monoglutamyl form (22).

4.3.4 Nitrous oxide

In humans, nitrous oxide (N_2O) irreversibly inactivates methionine synthase, and prolonged exposure results in megaloblastic anemia (49). Repeated exposures results in neurological problems such as subacute combined degeneration of the spinal cord (50). Inactivation was shown to occur only in the presence of all the components needed for MS turnover, and thus it was postulated that nitrous oxide intercepts the transient cob(I)alamin according to the following reaction:

cob(I)alamin + N_2O + H^+ ----▸ cob(I)alamin + N_2 + OH●

The generation of the hydroxy radical at the active site may explain the irreversible loss of activity (30).

4.3.5 Betaine homocysteine methyltransferase

This enzyme (EC 2.1.1.5) catalyzes the generation of methionine from homocysteine like MS. However, several lines of evidence have suggested that MS is more significant for the maintenance of tissue methionine. For example, MS is present in almost all mammalian tissues, and betaine methyltransferase has been found only in the liver and the kidney (51).

CHAPTER 5

INHERITED DEFECTS OF VITAMIN B_{12} METABOLISM

5.1 DEFECTIVE ABSORPTION OF Cbl

5.1.1 Defective Intrinsic Factor

Intrinsic factor (IF) is synthesized in the gastric parietal cell (63), and thus any conditon which destroys the gastric mucosa would result in a deficit of IF. The usual cause of an IF problem is the immunological destruction of the gastric mucosa, and this causes pernicious anemia. There are also cases of patients with normal IF secretion and abnormal Cbl absorption; when Cbl is mixed with normal gastric juice the absorption becomes normal. In some patients, this problem has been demonstrated to be the result of an abnormal IF protein with lowered affinity for the ileal receptor (64). This may also be the result of the secretion of a labile IF protein with a lowered affinity for Cbl (65).

5.1.2 Defective Cbl transport by enterocytes

Many cases have been reported of patients with abnormal Cbl absorption with a normal secretion of a functional IF protein, normal TCII levels, normal intestinal and gastric morphology, and no antibodies against IF in the serum. These cases have also not been corrected by the addition of normal IF (22). Symptoms include megaloblastic anemia, serum Cbl deficiency, and many patients also exhibit proteinuria. In some patients the problem has been described as a defect in or absence of the ileal cell IF-Cbl receptor; this has been referred to as the Immerslund-Grasbeck syndrome (68). Some patients, however, have been shown to have normal receptor activity, and the defect probably rests within the ileal cell itself (22).

5.1.3 Pancreatic insufficiency

Pancreatic insufficiency could cause Cbl malabsorption due to the lack of tryptic digestion of the R binders and the subsequent release of Cbl to allow binding to IF. Diseases of the small bowel, due to destruction of the absorption site, could also result in Cbl malabsorption (22).

5.1.4 TCII DEFICIENCY

Patients with this condition are usually normal at birth, with symptoms typically occurring in the first two months of life. Symptoms usually consist of vomiting, pallor, weakness, pancytopenia, or megaloblastic anemia, and neurological disease a few months after the initial manifestations. TCII has been demonstrated to be absent from these patients plasma when tested for Cbl binding capacity. These observations suggested that TCII synthesis by enterocytes was probably required for IF mediated transport of Cbl across the ileal cell (22).

5.1.5 R-Binder Deficiency

Only a few cases have been described with this condition, but there has been no evidence of adverse effects. The serum Cbl is usually in the deficient range, but the TCII levels are normal (22).

5.2 DISORDERS OF Cbl UTILIZATION

5.2.1 Genetic complementation groups

Different classes of mutations in the intracellular utilization of Cbl have been delineated through the use of tissue culture complementation assays. In the MCM branch

of the pathway, a complementation test was developed to analyze Sendai virus-induced heterokaryons formed from mutant pairwise mixing of mutant fibroblast lines (70). this was based on the report by Hill and Goodman (71) that fibroblast monolayers incorporated ^{14}C from (^{14}C)propionate into trichloracetic acid-precipitable material Polyethylene glycol (PEG) was later used as a fusing agent (72). If the uptake of label was increased in fused cells versus unfused cells, it was said that the two cells lines complemented each other, and presumably represented defects at two different genetic loci. There was always absence of intraclass and self-complementation. Another complementation test was developed to analyze defects in the methionine synthase branch of the Cbl pathway. This test measured the incorporation of (^{14}C) from (^{14}C)MeTHF into acid-precipitable material via synthesis of (^{14}C)methionine (72).

5.2.2 Methylmalonic aciduria (MMA)

Methylmalonic acid and its immediate precursor, propionate, are detectable in normal human blood, urine and cerebral spinal fluid only in small amounts. In the urine, the normal amount of methymalonic acid excreted is in the range of less than 15-20 µg/g creatinine. In methylmalonic aciduria, the levels of methylmalonic acid excreted can reach levels as high as 100mg to even 2g per g of creatinine (22). MMA is an inherited disorder which presents in patients as elevated levels of methylmalonic acid, ketones, and glycine in the blood and urine. This usually results in metabolic acidosis, ketosis, and hyperammonemia. Approximately half of the patients may also have hematologic abnormalities, despite experiencing normal serum Cbl levels (73). MMA can result from genetic deficiency of the MCM apoenzyme, a nutritional deficiency of Cbl, or a genetic deficiency in the synthesis of the MCM coenzyme, adenosyl-Cbl (74). Benign,

asymptomatic methylmalonic aciduria has also been observed through routine newborn screening (75). Analysis of two sibs with benign MMA revealed a mutation involving the MCM apoenzyme, which illustrates the enormous heterogeneity in clinical presentation of this disorder.

The classification of MMA is based largely on complementation analysis.

5.2.3 Defects in Methylmalonyl CoA mutase

The **mut** class represents patients with a defective or absent activity of the enzyme methylmalonyl CoA mutase and can affect as many as 1/29,000 newborns (75). Patients with this disorder are usually normal at birth, and develop symptoms rapidly with protein feeding. As in the cblA and cblB groups (see 5.3.1), symptoms can include MMA, lethargy, failure to thrive, vomiting, and others due to the accumulation of methylmalonic acid and the resulting metabolic acidosis, ketosis, and death. Although the clinical symptomatology of cblA, cblB and mut are similar, cblA and B tend to be less severe (76).

Mut patients have been classified into two groups based on the activity of their MCM. Mut^0 patients have no detectable mutase activity in their fibroblasts as measured by (^{14}C)propionate incorporation, and no detectable MCM activity in their cell extracts. Amongst mut^0 patients, some have demonstrated no protein synthesized, some have synthesized unstable protein, and one case has demonstrated a mutation that interferes with mitochondrial import of the premutase protein. Another line was discovered that carries a mutation that prevents normal mitochondrial processing of the premutase protein (77).

The second group of mut is called mut^-, and these patients demonstrate residual mutase activity when large amounts of adenosyl-Cbl are added to their intact fibroblasts.

Some patients have shown an MCM protein that has a lowered affinity for AdoCbl (76).

5.3 DISORDERS OF Cbl METABOLISM

Through complementation analysis, seven distinct groups of mutants of Cbl metabolism have been identified and defined alphabetically as cblA through G (22).

5.3.1 CblA and cblB

CblA and cblB represent two different complementation classes that have a defect in the synthesis of the coenzyme adenosyl-Cbl. Both defects usually present with methylmalonic aciduria. Symptoms usually arise within days after birth to 1 year of age, and consist of (in addition to MMA), lethargy, failure to thrive, vomiting, dehydration, respiratory distress, and developmental retardation, among others. These symptoms are related to the accumulation of methylmalonic acid in the plasma, with resulting metabolic acidosis, ketosis, and excretion of metabolite in the urine.

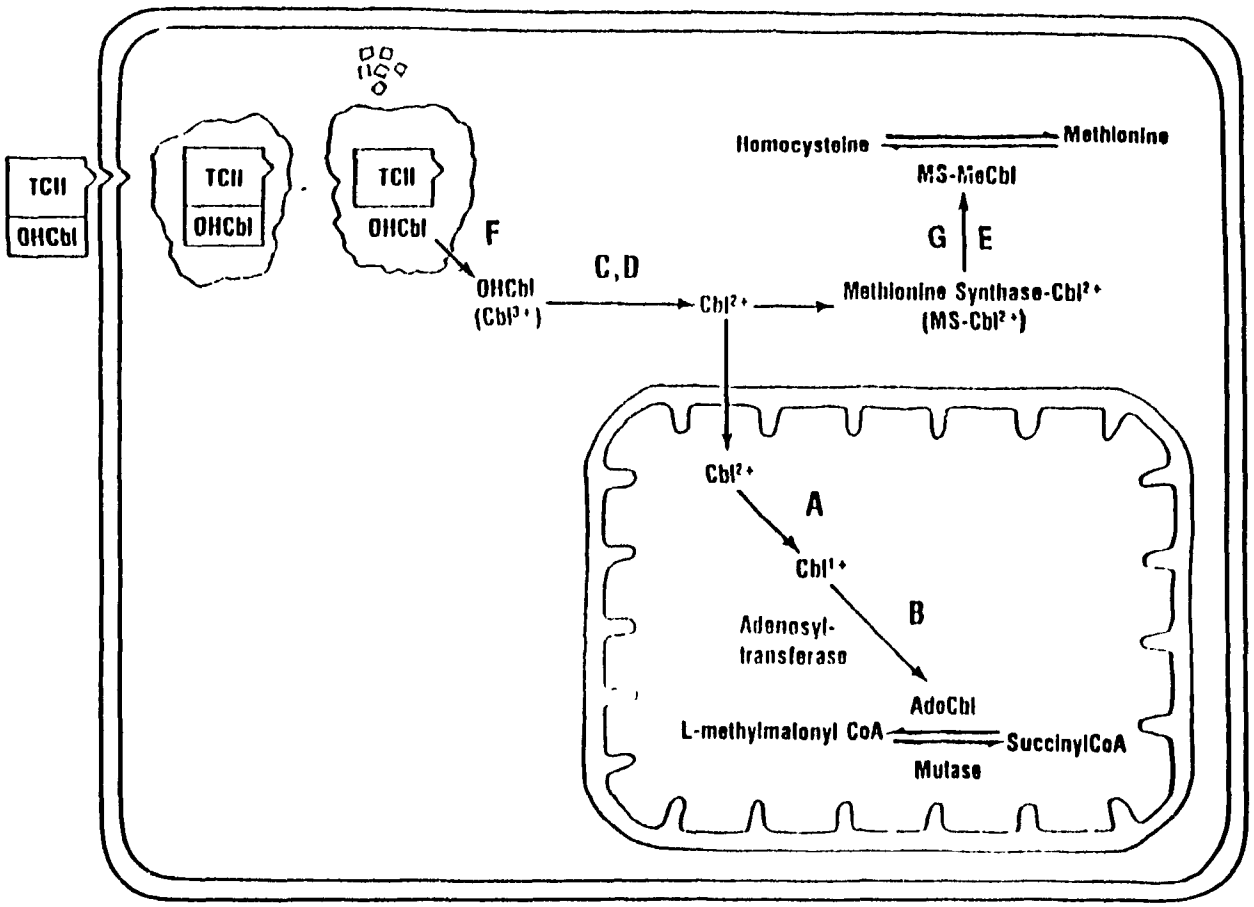
CblB has been determined to be the result of a defective or absent activity of the enzyme Cob(I)alamin:ATP-adenosyl transferase (EC 2.5.1.17), which synthesizes 5'deoxyadenosyl-Cbl from cob(I)alamin (77). Intact fibroblasts and cell extracts have shown deficient activity of this enzyme, and low levels of adenosyl-Cbl.

CblA patients have also shown abnormal synthesis of adenosyl-Cbl in their intact fibroblasts and low levels of adenosyl-Cbl, but their cell extracts have shown normal adenosyl-Cbl synthesis (77). The defect is as yet unknown, but it may involve the reduction of cob(III)alamin or cob(II)alamin to cob(I)alamin.

Most of these patients (roughly 70%) respond well to parenteral Cbl therapy, although cblA seems to respond better than the cblB group (22).

FIG.5 INBORN ERRORS OF VITAMIN B_{12} METABOLISM. The letters A through to G represent different classes of Cbl mutations at their putative sites along the Cbl pathway (cblA - cblG). Cbl = cobalamin; $Cbl^{1+,2+,3+}$ = cobalamin with 1+, 2+ or 3+ oxidation states of the cobalt core; OHCbl = hydroxocobalamin; MS-MeCbl = methionine synthase bound methylcobalamin; AdoCbl = 5'deoxyadenosylcobalamin; TCII = transcobalamin II. Reprinted from Rosenblatt and Cooper (110).

Inborn Errors of Vitamin B_{12} (Cbl) Metabolism



A third, related, complementation group (consisting of one patient) has been revealed recently (78) that behaves clinically and biochemically as a cblA. In addition to a similar clinical picture that includes MMA and ketotic hyperglycinemia, intact fibroblasts have demonstrated low levels of adenosyl-Cbl, and low MCM activity. Cell extracts demonstrated a normal MCM activity. This cell line, however, complemented with cblA and cblB cell lines. This complementation group has been tentatively called cblA', and demonstrates that cblA may affect at least two genetically different abnormalities. This indicates that at least two gene products in addition to adenosyltransferase are required to generate adenosyl-Cbl in the mitochondrion (78).

5.3.2 CblC and cblD

CblC and cblD patients usuallly exhibit homocystinuria and methylmalonic aciduria, hypomethioninemia, and megaloblastic anemia, due to a defect in the synthesis of both Cbl coenzyme forms, adenosyl-Cbl and methyl-Cbl.

CblC patients exhibit neonatal onset of failure to thrive, feeding difficulties, and developmental delay. Some patients had hypotonia, microcephaly, and seizures. Most had megaloblastic anemia, and their serum folate and Cbl levels were normal or elevated. Some patients have died, and the rest remain permanently impaired, despite treatment with OHCbl (22). CblC cells exhibit low levels of adenosyl-Cbl and methyl-Cbl, and total Cbl accumulation is only about 1/3 that of controls (79). Studies using gel filtration and polyacrylamide gel electrophoresis demonstrated a complete deficiency in binding of labelled Cbl to any Cbl-dependent apoenzymes (80). Cellular uptake of Cbl studies, however, have shown that cblC cells are able to take up Cbl-bound TCII into endosomes and hydrolyze the complex to the same degree as control cells (81). Extracts of these

mutant cells have also shown an ability to synthesize adenosyl-Cbl from chemically reduced Cbl (82).

CblC disease exhibits some heterogeneity, in that there is another group of cblC patients which exhibit late onset disease, which presents with a very mild form of the illness. Treatment of these patients with OHCbl appears to arrest and reverse the abnormalities, and leaves the patient with very little deficit (76).

CblD, which is comprised of only two brothers, resembles late onset cblC disease. The original proband presented at age 14 with behavioural problems and mild mental retardation, and was subsequently shown to have MMA. At the time of his diagnosis, his two year old sibling was asymptomatic (67).

Cells of cblD patients have been shown to exhibit some holoMS and holoMCM activity (80). Polyacrylamide gel electrophoresis has also demonstrated binding of radioactive Cbl to methionine synthase, and a very small amount to MCM. Compared to the highest cblC values, cblD cells showed propionate fixation and 5-MeTHF fixation at 50% and 100% higher respectively (80). CblD cells have also shown an ability to remove the CN group from Cbl at a level intermediate that of cblC (totally deficient in this ability) and control (43).

5.3.3 CblF

To date, two patients have been discovered that have cblF disease. The original proband presented with stomatitis, glossitis, convulsions, and neonatal Cbl-responsive MMA. Fibroblasts from this patient showed an abnormality in adenosyl-Cbl and methyl-Cbl synthesis, although this patient never exhibited homocysteinuria or megaloblastic anemia. Fibroblasts demonstrated a severe deficiency in both holoenzyme activities, and

low degree of Cbl responsiveness. Total Cbl uptake, however, was higher than that of controls. Cellular fractionation studies showed that most of the Cbl in these cells were in the lysosomes and were present as CNCbl. Thus the defect in cblF cells seems to be in the efflux of Cbl out of the lysosome (83).

5.3.4 CblE and cblG

Both cblE and cblG mutant classes possess a functional defect in the synthesis of methionine, a reaction that is catalyzed by methionine synthase. Patients from both groups may present, usually by the age of two years, with vomiting, poor feeding, developmental delay, and neurological problems ranging from visual deficits to seizures, hypo or hypertonia, to microcephaly (22,85). Patients also tend to exhibit homocystinemia and homocystinuria, megaloblastic anemia, and hypomethioninemia. All these symptoms respond to some degree to OHCbl treatment, with the neurological deficits being the most refractive to treatment. One case of cblE was diagnosed prenatally (an older sibling was already diagnosed with cblE disease (86)), and was treated in utero and post natally, has since shown no clinical symptoms, and has developed normally (87). This patient's fibroblasts are not distinguishable from other cblE fibroblasts in culture.

Most cases exhibited no methylmalonic aciduria, but there was one case of cblG disease with transient MMA (88). Symptoms from these two classes are extremely heterogeneous, and do not distinguish cblE from cblG. One cblG patient in fact presented in adulthood with ataxia, and this patient's fibroblasts are not distinguishable from other cblG fibroblasts in culture (84). Despite the clinical similarities, cblE and cblG belong to two separate complementation groups, and thus may represent defects at two separate genetic loci (89).

Fibroblast cultures from all these patients tend to exhibit poor growth in media where homocysteine replaces methionine, normal Cbl uptake and accumulation, normal adenosyl-Cbl levels and low methyl-Cbl levels. Methyl-Cbl synthesis in cblG patients tends to be less than in cblE patients, but an overlap does exist (85). Polyacrylamide gel studies have also shown normal Cbl binding to both enzymes in cblE and cblG cell extracts (86,46).

Methionine synthase assays performed on fibroblast extracts from cblE and cblG patients yielded another area of difference between these two classes. CblE intact cell methionine synthase activity, measured indirectly by the incorporation of 5-(^{14}C)MeTHF into acid-precipitable material, yielded low activities when compared to control activities, and was responsive to the addition of OHCbl to the assay (86). Cell extracts, however, demonstrated normal methionine synthase activity under standard reducing conditions (150mM 2-mercaptoethanol). CblE extracts would only exhibit lower enzyme activity than control extracts under substandard reducing conditions. Mixing of cblE extracts and control extracts under suboptimal reducing conditions were shown to correct the defect in enzyme activity. From these results, the defect in the cblE mutant was postulated to involve the maintenance of Cbl bound to MS in a reduced state; the defect would possibly affect a methionine synthase-associated reducing system (86). Such a system has been demonstrated to exist in bacteria (see section 4.1), but as yet, no such system has been demonstrated for the mammalian enzyme. Another observation to substantiate this theory is the fact that N_2O cannot inactivate MS in cblE cells, as it does in control cells (see section 4.3.4). N_2 has been postulated to react with Cbl^{1+} bound to the MS enzyme during turnover, so thus if Cbl^{1+} cannot be generated, N_2O cannot inactivate the enzyme (86).

CblG fibroblasts have usually exhibited low methionine synthase activity in intact cells and in cell-free extracts under standard reducing conditions, leading to the conclusion that the cblG defect does not involve a MS-associated reducing system (85). Recently, however, extracts from a cblG cell line have shown normal enzyme activities under certain assay and growth conditions, exhibiting the heterogeneity of this defect (46). These same studies also demonstrated that in this and one other CblG cell line, the methionine synthase activity was sensitive to the concentration of AdoMet in the assay. Where control cells exhibited inhibition of MS activity in the presence of a large concentration of AdoMet, cblG cells showed no such inhibition; these cblG cells also required a higher concentration of AdoMet to exhibit normal enzyme activity. From these observations, the defect in cblG disease was postulated to involve the manner in which AdoMet associates with MS-bound Cbl. It has also been postulated that cblG disease may result from a defect in the MS protein itself (89).

MCM activity was also measured in intact cblE and cblG fibroblasts by the incorporation of label from (^{14}C)propionate into acid-precipitable cellular macromolecules. Not all cell lines showed normal activity, but the levels were never as low as those seen in mut, cblA, cblB, cblC or cblD mutants.

CblE and cblG disease are both thought to be autosomal recessive disorders. Parents of the original cblE proband were examined for their synthesis of methyl-Cbl and intact cell methionine synthesis, and their values were found to be intermediate to the affected child and control values (87).

CHAPTER 6

MATERIALS AND METHODS

6.1 MATERIALS

Cell culture media was purchased from Flow Laboratories, Miss.Ont.; Fetal Bovine Serum (FBS) was purchased from Gibco Laboratories, Grand Island, N.Y., and Bocknek Laboratories, Rexdale,Ont.; 5-[^{14}C]methyltetrahydrofolate and [^{57}Co]CNCbl from Amersham Corp., Oakville, Ont.; S-adenosylmethionine, DL-homocysteine thiolactone, 5-methyltetrahydrofolate, and methyl-Cbl from Sigma Chemical Co., St. Louis,MO; Bio-Rad AG1X8 resin, 200-400 mesh, chloride form, acrylamide, bis, riboflavin and temed from Bio-Rad Laboratories, Richmond,CA; Betamax scintillation cocktail from ICN, Irvine,CA.; and Formula 963 scintillation fluid and Protosol from New England Nuclear, Boston, Mass.

6.2 METHODS

6.2.1 Cell culture

Skin fibroblasts were obtained with informed consent from patients with methyl-Cbl deficiency. Fibroblasts were stored at the Repository for Mutant Human Cells, Montreal Children's Hospital (Montreal, Quebec). All cell strains were determined to be free of mycoplasma contamination by a modification of the method of Schneider et al (109). Cultures were routinely maintained in 175 cm^2 flasks (Falcon, Oxnard, California), and were fed twice a week with Eagle's minimum essential medium with Earle's salts, L-glutamine, and nonessential amino acids (MEM) supplemented with 10% (vol/vol) fetal bovine serum.

TABLE 1. CELL LINES USED IN THIS STUDY. MCH refers to control lines obtained from the Montreal Children's Hospital Mutant Cell Repository, and WG refers to mutant cell lines. Trypsinization refers to the number of passages a culture has passed through.

TABLE 1. **CELL LINES**

CELL LINE	MUTANT GROUP	CELL CULTURE GENERATION	CELL CULTURE TRYPSINIZATION
MCH 39	control	25.5-29.5	15-20
MCH 45	control	23.5-27	18-21
MCH 65	control	10.5-16	5-8
WG788	cblE	x+17.5-x+22	x+13-x+17
WG1146	cblE	11.5	7
WG1296	cblE	x+14.5	x+14
WG1384	cblE	x+11.5	x+13
WG1663	cblE	x+5	x+3
WG1401	cblE	15.5	15
WG1575	cblE	14	12
WG1205	cblG	x+10.5	x+10
WG1223	cblG	14.5-15.5	9-10
WG1308	cblG	13.5-31	8-22
WG1386	cblG	x+8	14
WG1408	cblG	11-13.5	5-8
WG1505	cblG	32	16
WG1595	cblG	x+8-x+9	x+6-x+8
WG1655	cblG	x+10.5-x+12	12-15
WG1670	cblG	x+13.5-x+18	12-15
WG1671	cblG	x+8-x+9.5	7-8

Cultures were kept at 37^0 C in $95\%CO_2/5\%O_2$. Cultures of confluent fibroblast monolayers were harvested by washing cell surface with PBS buffer (PBS consists of, in Molar concentrations: NaCl (1.37), KCL ($3.0x10^2$), Na_2HPO_4 ($8.0x10^{-2}$), KH_2PO_4 ($6.0x10^{-3}$), glucose ($6.0x10^{-2}$), and chloroform (0.2% v/v)) three times. Cells were then released from the flask by incubating then with 0.25% trypsin for about 10 minutes at room temperature. Trypsinization was terminated by the addition of culture media. Cells were then washed with PBS and the pellet was resuspended in an appropriate buffer, as described below.

6.2.2 Polyacrylamide gel electrophoresis

Fibroblasts were incubated in 50 pg/ml [^{57}Co]CNCbl + 10% human serum as a source of TC II for 5 days, trypsinized and then resuspended in 600 or 800 µl of 0.25M sucrose and 0.02M Tris HCl pH 7.4 (cells were resuspended in 800µl of buffer if the harvesting yielded over $50x10^6$ cells). The cell suspension was sonicated on ice 3x30sec blasts at 12µ and the sonicate was centrifuged at 5° C at 245,000g for 30 min. The resulting supernatant was stored at -85^0 C until used for electrophoresis. An aliquot was removed for determination of protein content (Lowry protein assay (105)).

Supernatants were diluted by 10% with 2% Triton (Sigma Chemical Co., St. Louis, MO) to help break up any residual membranes, prior to application to the acrylamide gel. A native 4% (29:1 acrylamide/bis) horizontal slab polyacrylamide gel (pH8.9) was poured the day before the electrophoresis, and was allowed to photopolymerize overnight in the presence of fluorescent light, with riboflavin and temed as the initiators of polymerization. Extracts were run in duplicate for 4 hours at 45 mA, after which the gel was frozen, and then cut into 2mm slices. The slices were incubated overnight at 37^0 C in Betamax

scintillation cocktail with 3% Protosol to extract radioactivity. The samples were then analyzed in a scintillation counter (Beckman Instruments, Miss. Ont.).

6.2.3 Methionine synthase assay

Confluent fibroblast cultures were trypsinized and resuspended at a density of 1-2 x 10^8 cells/ml in 0.25M Sucrose. This cell suspension was sonicated on ice, 4x30 sec blasts at 12μ and spun at 5^0 C at 170,000g for 45 min. The resulting supernatant was stored at -85^0 C. At the time of assay, the cell extract was diluted by 10% with a 1M potassium phosphate buffer pH7.4 to form a cell extract in 0.1M KPhos buffer pH7.4. An aliquot of this final solution was removed to determine protein content (Lowry protein assay (105)).

Enzyme activity was measured as previously described (89). The assay mixture for measurement of holoenzyme activity contained: varying amounts of cell extract and 0.1M KPhos for a total volume of 100μl; 100mM KPhos buffer, pH7.4; 250μM S-adenosylmethionine; 500μM DL-homocysteine (prepared fresh daily from the thiolactone); 150mM 2-mercaptoethanol; and 390μM 5-[^{14}C]methyltetrahydrofolate(1.4dpm/pmol); for a total assay volume of 200μl. For assay of total enzyme activity 50μM of methyl-Cbl was added.

The assay mixture was incubated in the dark at 37^0 C in stoppered vacutainer tubes that had been flushed with nitrogen for 7 sec. The reaction was stopped with the addition of 800μl of ice cold distilled water, and the reaction mixture was applied to a syringe mini-column with a bed volume of 1.5ml Bio-Rad AG1X8, 200-400 mesh, chloride form resin (Bio-Rad Laboratories). The reaction tubes were washed with another 1ml of water which was applied to the columns. The resulting effluent of 2ml was then

analyzed for radioactivity in a Beckman scintillation counter, after the addition of 3ml of NEN formula scintillation cocktail, number 963.

6.2.4 Fractionation of intracellular cobalamins

Washed cell pellets of cells grown in 25pg/ml of labelled cobalamin were extracted in complete darkness in 10 ml of absolute ethanol at 85^0 C for 20 min and centrifuged, and 8 ml of supernatant ethanol was evaporated to dryness under a stream of nitrogen at room temperature. The remaining 1 ml aqueous sample was analyzed on high pressure liquid chromatography using a Merck column Lichrosorb RP-8 (E.Merck, Darmstadt, West Germany), 10μm eluted with a gradient of phosphate at pH 3 and triethylammonium phosphate (2). Fractionation studies were performed by Sally Luc-Shing in the lab of Dr B.A. Cooper of McGill University and the Department of Haemotology, Royal Victoria Hospital.

CHAPTER 7

RESULTS

7.1 Polyacrylamide gels:

As reported previously, in fibroblasts incubated for 5 days in [^{57}Co]CNCbl, over 95% of the B_{12} found in cells is bound either to methionine synthase or methylmalonyl CoA mutase (90).

Figure 6 shows profiles of 4 different cell lines run on a 4% native polyacrylamide gel. These lines were incubated for 5 days in 50pg/ml [^{57}Co]CNCbl with 10% human serum as a source for TCII. MCH39 represents a control line (top left, Fig.6) and shows two peaks of [^{57}Co]CNCbl binding. From other studies it has been determined that the smaller peak represents methylmalonyl CoA mutase and the larger peak represents methionine synthase (80,86).

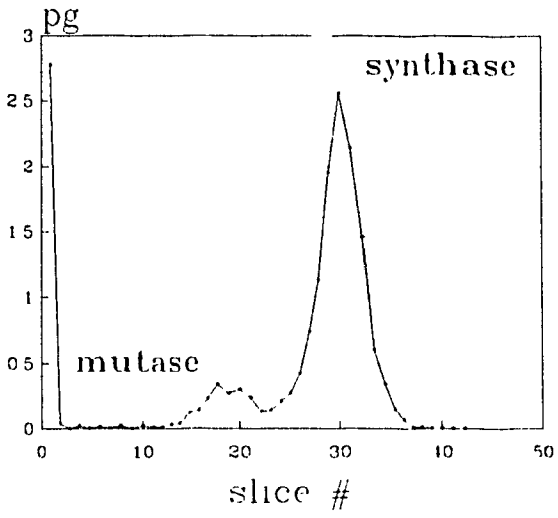
It has also been determined that TCII-B_{12} (whose peak falls in between the two enzyme peaks) does not contribute significantly to either peak (80).

For cell line MCH39 (Fig. 6), 13% of the B_{12} bound to the two peaks resides in the smaller peak, the mutase enzyme, and the remaining 87% of the counts resides in the larger peak, the synthase enzyme.

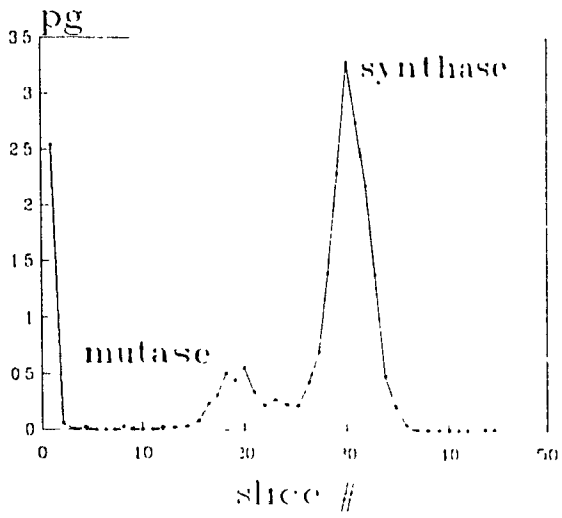
Cell line WG788 (top right, Fig.6) represents a typical cblE line, and shows a binding distribution equal to that of control lines, with 14% of the label bound to the mutase peak and 86% bound to the synthase peak. All seven cblE lines showed this kind of distribution.

FIG.6 **POLYACRYLAMIDE GEL PROFILES OF A CONTROL, A cblE, AND TWO cblG CELL LINES.** Cell lines were incubated with [^{57}Co]CNCbl for 5 days, and cell extracts were run on a 4% native polyacrylamide gel as outlined in Methods. WG1671 represents 1 of 3 cblG cell lines which show no association of Cbl with MS.

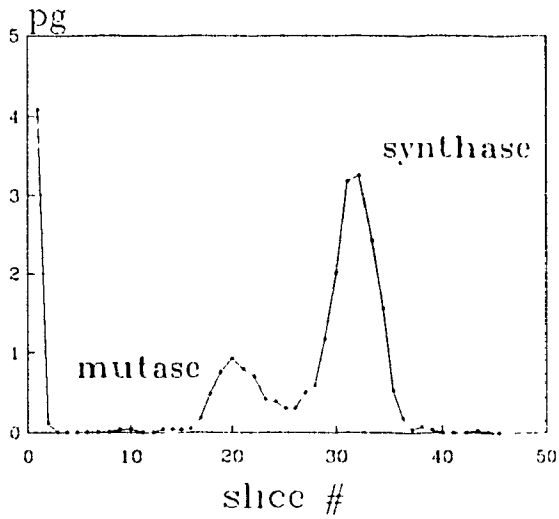
MCH39
control



WG788
cblE



WG1223
cblG



WG1671
cblG

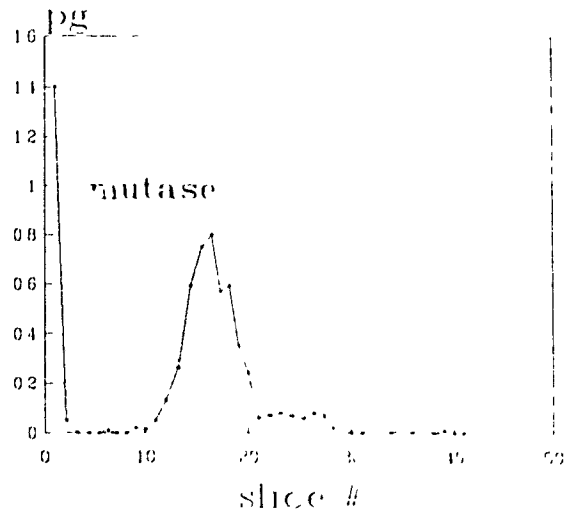
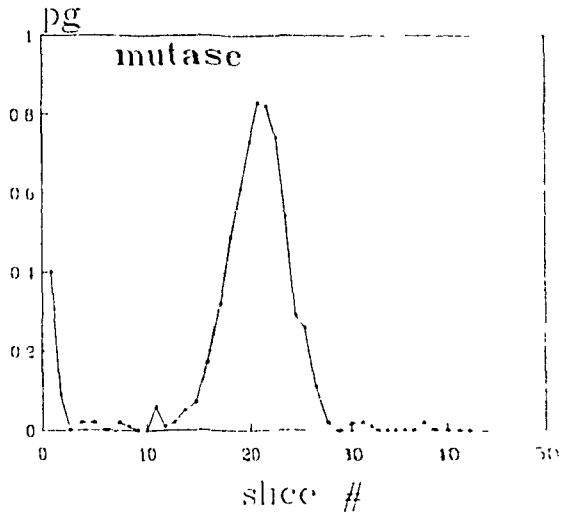
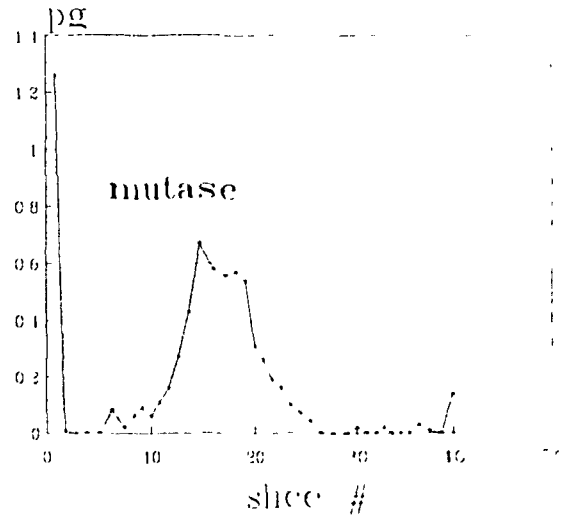


FIG.7 **POLYACRYLAMIDE GEL PROFILES OF 2 cblG CELL LINES WHICH SHOW NO ASSOCIATION OF Cbl WITH METHIONINE SYNTHASE.** Two of three cblG cell lines which show no binding of Cbl to MS. WG1670 is a sibling of WG1671 (Fig. 6).

WG1655
cblG



WG1670
cblG



Cell line WG1223 (bottom left, Fig.6) represents a majority of cblG lines which also show binding distributions close to that of control lines. In this particular case, 24% of the label was bound to the mutase and 76% was bound to the synthase. Seven out of ten cblG lines showed this kind of distribution.

Cell line WG1671 (bottom right, Fig.6) however, showed little or no binding of vitamin B_{12} to the methionine synthase peak. Three out of ten cblG lines, two which are siblings, showed this lack of binding. The other two nonbinding cell lines, WG1670 (sibling of WG1671), and WG1655 are pictured in Fig.7.

As the profiles indicate, there was a large peak of radioactivity evident in the first slice of the gel. This represents material that was held up in the well and did not enter the gel. First slice counts varied not only between cell lines but also varied from gel to gel within cell lines. I performed regression analyses to see if there was any correlation between the amount of radioactivity in the first slice and the amount in the mutase or synthase peak. For all controls, and all cblE cells, the correlation was close to zero with the both peaks. In the seven nonbinding cblG lines, there was a negative correlation of 0.45 with the mutase peak, and a positive correlation of 0.45 with the synthase enzyme. This indicates that there is a higher probability that the first slice counts are varying inversely with the mutase peak, i.e. when one goes up the other goes down. This may be a coincidence, but it also may indicate that mutase is being held up in the well for the cblG lines. Since my observations are more qualitative that quantitative, i.e. if B_{12} binds or does not bind to the synthase enzyme, I feel that it does not affect the final result.

These results are summarized in Table 2. Accumulation of label in cblE cells and in 7 out of 10 cblG cells was equal to that of control cells. In 3 cblG lines, the uptake,

while still substantial, was lowered (Table 2).

These results illustrate that most cblG cell lines do bind vitamin B_{12} to methionine synthase despite the functional deficiency. The results also show heterogeneity within the cblG group which manifests as a lack of binding to the methionine synthase enzyme.

The vitamin B_{12} distributions were also examined in each cell extract using High Performance Liquid Chromatography (Table 3); this assay was performed in Dr. B.A.Cooper's laboratory at McGill University by Sally Lue-Shing. These distributions represent cobalamin forms from which the protein was extracted. The proportion of labelled CNCbl converted to methyl-Cbl was decreased compared to that of controls in all cblE and cblG cell lines (Table 3). This was associated with increases in other forms, especially adenosyl-Cbl. In the three cblG lines which showed no association of Cbl with methionine synthase, there was a greater decrease in methyl-Cbl, an even higher proportion of Cbl as adenosyl-Cbl, and a very low amount of Aq-Cbl.

TABLE 2. ENZYME-BOUND COBALAMIN IN CONTROL, cblE AND cblG FIBROBLASTS. [^{57}Co]-CN-Cbl was preincubated with 10% human serum at 37^0 before incubation with cells. The final CNCbl concentration was 37 pM. Cells were incubated for 5 days as outlined in methods. Results are shown as means $\pm$ standard deviations.

Table 2. Enzyme-Bound Cobalamin

Cell line	uptake(pg/mg prot.)	%mutase	%synthase
control (n=3)	48.8 ±4.2	15.7 ±2.9	84.3 ±2.9
cblE (n=7)	52.1 ±26.8	16.8 ±3.6	83.2 ±3.6
cblG (n=7)	49.2 ±18.3	25.5 ±5.4	74.5 ±5.4
cblG (n=3)	29.9 ±10.6	100	0

TABLE 3. COBALAMIN DISTRIBUTIONS IN WHOLE CELL EXTRACTS IN CONTROL, cblE AND cblG FIBROBLASTS. Fibroblasts were incubated for 4 days at 25pg/ml [^{57}Co]-CN-Cbl. Extracts had protein removed and were processed via HPLC in the dark as described in Methods. From left to right are: aquo-Cbl, cyano-Cbl, adenosyl-Cbl, methyl-Cbl, and other forms which occur in small amounts. Standard deviations appear in brackets.

Table 3. Cobalamin Distributions

	Aq%	CN%	Ado%	Me%	Others%
control	8.3	9.4	18.1	52.6	11.4
(n=5)	(3.7)	(5.8)	(3.2)	(12.5)	(6.4)
cblE	24.2	19.0	23.2	11.4	21.1
(n=7)	(9.2)	(15.2)	(8.0)	(6.8)	(6.1)
cblG	30.7	12.8	31.7	7.0	17.8
(n=7)	(5.5)	(4.6)	(5.5)	(1.8)	(8.6)
cblG	3.8	10.3	62.9	3.5	19.7
(n=3)	(3.2)	(2.9)	(9.7)	(2.0)	(7.8)

7.2 Methionine synthase assays:

All the synthase assays were performed under standard reducing conditions (150mM 2-mercaptoethanol). Under these conditions, cblE cells show methionine synthase activities equal to that of control extracts (86).

I have performed synthase assays on one cblE line (WG1575), one cblG line that does bind cobalamin to methionine synthase (WG1308), and all three nonbinding cblG lines (WG1655, WG1670, and WG1671) (Table 4). I chose to assay only one binding cblG line as a representative of that group; five out of seven binding cblG cell lines have previously been assayed in this laboratory (85) and all five yielded similar levels of activity.

As expected, cell line WG1575 has a rate of methionine synthase activity equal to that of control cells (Table 4). Under these conditions, however, the binding cblG line (WG1308) shows a rather low level of synthase activity, and the three nonbinding lines exhibit extremely low levels of activity.

TABLE 4. METHIONINE SYNTHASE ACTIVITY IN FIBROBLAST EXTRACTS FROM A CONTROL, A cblE, AND FOUR cblG CELL LINES. Cell extracts were prepared and methionine synthase activity was determined as outlined in Methods. Methionine biosynthesis was determined in the presence of methyl-Cbl (i.e. total enzyme activity). CblG(v) represents those cblG lines which show no association of Cbl with methionine synthase.

Table 4. Methionine Synthase Activity in Fibroblast Extracts

cell line	protein added (mg)	methionine formed (pmol/min)
MCH39-control	0.4	42.8
WG1575-cblE	0.3	44.8
WG1308-cblG	0.4	3.6
WG1655-cblG(v)	0.6	0.4
WG1670-cblG(v)	0.6	0.4
WG1671-cblG(v)	0.8	0

CHAPTER 8

DISCUSSION

This thesis has investigated the patterns of vitamin B_{12} binding in cell extracts from cblG patients in order to see if the lowered activity of the methionine synthase enzyme is reflected in decreased binding of Cbl to the enzyme. I discovered that for most cblG patients, there is a substantial amount of Cbl bound to methionine synthase, and thus there appears to be no defect in cofactor association with methionine synthase in the cblG mutant group. However, I have also uncovered heterogeneity within the cblG class which is manifested by a lack of association of Cbl with the methionine synthase enzyme.

The original proband with a functional defect in the methionine synthase system was reported by Rosenblatt et al. in 1984 (86). This patient was later assigned to a new complementation group within the inherited disorders of Cbl metabolism and named cblE. This defect is believed to involve a methionine synthase-associated reducing system, as outlined in section 5.3.4. In 1988, Watkins and Rosenblatt (89) reported on another infant which presented much in the same manner as cblE patients do, and this patient was believed to belong to this mutant class. Complementation studies, however, yielded the information that while this patient did indeed have a functional defect in the methionine synthase system, it was not the same defect as in the cblE patients. This new group, which complemented cblE cells in culture, was called cblG. It was also noted that while cblE cell extracts showed normal methionine synthase activity under standard reducing conditions, cblG cells consistently showed very low levels of activity. From these observations, it is believed that cblE and cblG represent defects at two different genetic

loci (89).

In my experiments, I used cell extracts of control, cblE and cblG patients to see if I could uncover some difference in cofactor binding between these three groups. In all, I used 3 control, 7 cblE and 10 cblG cell lines. I obtained the result that all cblE, and 7 out of 10 cblG lines showed a normal association of Cbl with the enzyme methionine synthase, while three lines showed a lack of association of vitamin B_{12} with the enzyme (Figs. 7 and 8) (Table 2).

Previous studies have not consistently examined the association of Cbl with methionine synthase in cblG or cblE patients. Those that have examined the association have always shown a pattern like that of control cells, with at least three quarters of the intracellular Cbl bound to methionine synthase and the remainder bound to methylmalonyl-CoA mutase (86) (46). At least 95% of all the Cbl found intracellularly is bound to either of these two enzymes (90). In this study, I report an average of 85% of intracellular Cbl bound to methionine synthase, and the remaining 15% bound to the mutase enzyme in control cells (Table 2).

Further evidence that confirms the heterogeneity of these 3 cblG variant cell lines may be seen in the B_{12} distributions in cells (Table 3). The Cbl distributions were obtained by running cell extracts, from which the protein had been removed, in the dark on an HPLC. As expected, cblE cells showed very low amounts of methyl-Cbl in their cells, with resultant increases in the other Cbl forms, namely AquoCbl, CNCbl, adenosyl-Cbl, and other forms which occur in small amounts (Table 3). The 7 cblG cell lines that do show association of Cbl with methionine synthase had a level of methyl-Cbl similar to the level in cblE cells. The cblG variant cells, however, had a methyl-Cbl level that is close to zero (significantly lower than the 7 cblG and cblE cell lines, $p=0.05$), and also

had a higher proportion of adenosyl-Cbl than all the other cell lines. These results not only support the observations that these 3 cell lines have a severe defect in the methionine synthase enzyme, but also suggest that the Cbl that is not binding to MS in the cytoplasm is moving into the mitochondrion where it is being converted to adenosyl-Cbl. In fact, these cblG variant cell lines, though exhibiting a lower Cbl uptake than do the other cblG cell lines, do display an uptake that is higher than would be expected for cells which show no association of Cbl with MS. If roughly 85% of the intracellular Cbl is bound to the MS enzyme, then cells deficient in Cbl binding to MS would be expected to demonstrate roughly an 85% decrease in uptake. This has not been observed, and could be explained by the excess formation of adenosyl-Cbl. As of yet, however, it is not known to what degree the mutase enzyme may be stimulated by the presence of Cbl.

The cblG variant cell lines also exhibit a lower proportion of Cbl as AquoCbl than the other 7 cblG lines. This is presumably due to the fact that some of the Cbl bound to MS in the 7 cblG cell lines may be in the aqueous form (instead of the methyl form). The cblG variant cell lines do not bind Cbl to methionine synthase, and as previously stated, their Cbl is presumably being converted to adenosyl-Cbl in the mitochondria.

MS assays have been performed previously on many cblE and cblG cell lines. CblE cell extracts consistently show normal to high activity of MS under standard reducing conditions, while most cblG cell lines have demonstrated very low but detectable MS activity under standard reducing conditions (85). One study, however, yielded normal enzyme activities of a cblG line if it was grown under specific conditions (methionine and CN-Cbl added to the media) (46); this once again demonstrated that there is a large amount of heterogeneity in the way in which cblG cell lines may express their defect (as a point of interest, this particular cell line was one of the 7 cblG cell lines that showed

an association between Cbl and MS). Under the growth conditions in our laboratory (Eagle's minimum essential medium supplemented only with fetal bovine serum, as outlined in Methods), cblG fibroblasts have always shown very low specific activities. In this study, I performed MS assays on 1 control, 1 cblE, 1 cblG (whose MS binds Cbl), and the 3 cblG variant cell lines under standard reducing conditions (150 mM 2-mercaptoethanol). As expected, the cblE cell line demonstrated a normal level of MS activity, and the cblG cell line that shows association of Cbl with MS showed a very low level of activity (Table 4) (the MS activity in 5 out of 7 cblG cell lines has previously been assayed in this laboratory and all 5 have demonstrated a comparable level of activity to WG1308 (85)). The 3 variant cblG cell lines seemed to show an even lower amount of activity, with 1 line having no observable activity whatsoever. This may denote another area of heterogeneity between cblG and cblG variant cell lines.

Clinically, cblG patients tend to present with varying degrees of homocysteinuria, homocystinemia, hypomethioninemia, megaloblastic anemia, developmental delay, and a variety of neurological symptoms which may include seizures, mental retardation, hypotonia or hypertonia, lethargy, feeding difficulties, ventricular dilatation and cerebral atrophy (85). Patients usually present by the age of 2 years, although age and symptomology may be very heterogeneous. In fact, one cblG patient presented only at age 21 with ataxia (84). The 3 variant cblG patients presented in much the same manner as other cblG patients, but with a very early age of onset and they have seemed to remain somewhat more severely affected than other patients in the cblG group.

C.A. Hall in 1989 (46) observed that several cblG cell lines exhibited an altered state of sensitivity to the concentration of AdoMet in the MS assay. CblG cells needed more AdoMet to achieve normal MS levels of activity than control cells did, and these

cblG cells demonstrated a lack of inhibition of MS activity with a high level of AdoMet present. Control cells exhibit this inhibition. Based on these observations, Hall et al. proposed that the defect in cblG cells may rest in the manner in which AdoMet reacts with MS.

From the results of my study, I propose that the cblG defect rests within the apoenzyme itself (though I do not address whether this defect is infact associated with the AdoMet interaction with MS). These cblG variant cells were deemed to be in the cblG class through complementation studies, and thus may represent defects at the same genetic locus. The cblG defect which demonstrates an association of Cbl with methionine synthase may contain a defect in the manner in which the cofactor associates with the MS enzyme. The 3 cblG variant patients may not bind Cbl to MS due to a more severe expression of the same defect. Alternately, these cblG variant patients may also be expressing another mutation which results in a deficiency of the MS protein itself, and this would represent a new class of mutant in the Cbl pathway. If the MS protein is present and nonfunctional, or if the MS protein is absent may result in a lack of complementation between cblG and cblG variant cell lines, and thus would result in these two defects being classified in the same mutant group.

Further studies in this area should include establishing if the MS protein is present in the cblG variant cell lines. The MS protein has been purified, and the search for MS specific antibodies is continuing. Once the antibody is found, verifying the presence or absence of the enzyme will be simpler. Cloning the MS gene would then allow the delineation of the exact defect causing cblG, and perhaps, cblG variant disease Additional tissue culture complementation studies may also be performed between all the cblG and cblG variant cell lines to see if there is any further heterogeneity within the class.

SUMMARY

This study has investigated the patterns of Cbl association with the Cbl-dependent enzyme methionine synthase in 3 control, 7 cblE and 10 cblG cell lines. We have discovered that most cblG cell lines do show a significant amount of Cbl bound to methionine synthase despite the lack of enzyme activity. We have also uncovered heterogeneity within the cblG class of mutant which demonstrates a deficiency in association of Cbl with the enzyme. These cblG variant cell lines also exhibit higher levels of adenosyl-Cbl than in other cblG and all cblE cell lines, and demonstrate almost no methionine synthase activity. Due to these observations, we postulate that the defect in the cblG mutation rests in the apoenzyme itself.

CLAIMS TO ORIGINALITY

1) This thesis describes for the first time a variant of cblG complementation class associated with a lack of binding of Cbl to methionine synthase.

PUBLICATIONS

ABSTRACTS

Sillaots, S.L., and Rosenblatt, D.S., Vitamin B_{12} bound to target enzymes in cblE and cblG disorders. Presented at the Annual Meeting of the Canadian Society for Clinical Investigation in Toronto, Ont. Sept. 14-17, 1990.

Clin. Invest. Med. 13:B67 1990.

(CSCI Student Award winning presentation)

Sillaots, S.L., Hall, C.A., and Rosenblatt, D.S., Heterogeneity in cblG: Differential binding of vitamin B_{12} to methionine synthase. Presented in poster session at The American Society of Human Genetics Annual Meeting in Cincinnatti, Ohio, Oct. 16-20, 1990.

Am. J. Hum. Gen. 47:A166, 1990.

ARTICLE

Sillaots, S.L., Hall, C.A., and Rosenblatt, D.S., Heterogeneity in cblG: Differential Binding of Vitamin B_{12} to Methionine Synthase. Submitted to the American Journal of Human Genetics, December, 1990.

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