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Methylmalonicacidemia: Biochemical heterogeneity in defects of 5′-deoxyadenosylcobalamin synthesis*

(vitamin B_{12}/coenzyme metabolism/mitochondria)

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ABSTRACT **We measured the synthesis of 5′-deoxyadenosylcobalamin (AdoCbl) in fibroblast extracts from patients with inherited methylmalonicacidemia due to deficient activity of the cobalamin-dependent holoenzyme, methylmalonyl-CoA mutase (EC 5.4.99.2). Previous studies with intact fibroblasts from patients whose holoenzyme deficiency was secondary to abnormal cobalamin metabolism had defined two phenotypes, one in which whole cells failed to accumulate AdoCbl and a second in which they failed to accumulate both AdoCbl and the second cobalamin coenzyme, methylcobalamin. With a broken cell assay of AdoCbl synthesis, we have further subdivided the first phenotype into two classes. One class shows normal AdoCbl synthesis in cell extracts and the cell lines are named cbl A mutants; the other class shows severe deficiency of AdoCbl synthesis and the cell lines are named cbl B mutants. We define cbl C mutants as those in which both AdoCbl and methylcobalamin fail to accumulate in intact cells. The assay for AdoCbl synthesis is thought to measure two enzymatic activities, cob(II)alamin reductase (EC 1.6.99.9) and cob(I)alamin adenosyltransferase (EC 2.5.1.17). Subcellular fractionation studies place this combined activity in mitochondria.**

Inherited methylmalonicacidemia was first described in 1967 in infants who had retardation, recurrent vomiting, life-threatening attacks of ketoacidosis, and intolerance to high protein feedings (1, 2). All reported patients with this disorder but one (3) have had deficient activity of methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2) with a resultant block in the conversion of L-methylmalonyl-CoA to succinyl-CoA (Fig. 1). Deficient activity of this holoenzyme may be caused by a defective mutase apoenzyme or by deficiency of 5′-deoxyadenosylcobalamin (AdoCbl), the coenzyme necessary for mutase activity (4, 5).

The reactions leading to the intracellular synthesis of this coenzyme have been described in studies with cell-free extracts in bacteria (6, 7) and HeLa cells (8). The cobalt atom of cob(III)alamin is reduced in two steps, first by cob(III)alamin reductase [NADH:cob(III)alamin oxidoreductase, EC 1.6.99.8] to form cob(II)alamin and then by cob(II)alamin reductase [NADH:cob(II)alamin oxidoreductase, EC 1.6.99.9] to form cob(I)alamin (Fig. 1). Cob(I)alamin and ATP are substrates for cob(I)alamin adenosyltransferase [ATP:cob(I)alamin *Coβ*-adenosyltransferase, EC 2.5.1.17], the final enzyme required for the synthesis of AdoCbl. Such a sequence has not yet been elucidated for the synthesis of methylcobalamin (MeCbl), the other cobalamin coenzyme found in human tissues. MeCbl is utilized by 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13) in the simultaneous conversion of methyltetrahydrofolate to tetrahydrofolate and homocysteine to methionine. It is not known how many of the intracellular reactions leading to synthesis of each coenzyme are common to both.

Two discrete inborn errors of cobalamin metabolism as well as an abnormality of the mutase apoenzyme were identified previously with intact, cultured fibroblasts from patients with methylmalonicacidemia. Using intact fibroblasts grown for 2–5 days in culture medium containing the cob(III)alamin vitamin, hydroxo[^{57}Co]cobalamin (OH-Cbl), we measured the synthesis of radiolabeled AdoCbl and MeCbl (9). Intact fibroblasts from one group of patients with methylmalonicacidemia accumulated negligible amounts of AdoCbl but normal amounts of MeCbl (10). These cells had deficient mutase activity but normal methyltransferase activity. Many of these patients, in contrast to those with a mutase apoenzyme defect, show a beneficial clinical or biochemical response to pharmacologic doses of OH-Cbl or cyanocobalamin (11). Fibroblasts from other patients with methylmalonicacidemia failed to accumulate both AdoCbl and MeCbl; as a result, mutase and methyltransferase activities were impaired (10, 12). These patients have homocystinuria in addition to methylmalonicacidemia, and may also have a megaloblastic anemia (13). Fibroblasts from patients with a mutase apoenzyme defect were indistinguishable from control cells in the ability to accumulate cobalamin coenzymes.

In an attempt to define more precisely the specific biochemical abnormalities in cells with the above mentioned defects in cobalamin metabolism, we have adapted the cell-free assay for AdoCbl synthesis to crude extracts and subcellular fractions of fibroblasts. With this assay we have distinguished two distinct mutant classes in those fibroblast lines that share the intact cell phenotype of deficient AdoCbl accumulation and normal MeCbl accumulation.

MATERIALS AND METHODS

Cell Lines. Skin fibroblasts from seven normal individuals and 14 patients with methylmalonicacidemia were used in these studies. Eight of the methylmalonicacidemia lines (1f, 209, 214, 215, 221, 224, 245, 283) have the intact cell phenotype of failure to accumulate AdoCbl only. Two lines (78, 287) fail to accumulate both AdoCbl and MeCbl, and four lines (77, 216, 257, 288) are mutase apoenzyme mutants which accumulate both coenzymes normally.

Abbreviations: Cbl, cobalamin; AdoCbl, 5′-deoxyadenosylcobalamin; MeCbl, methylcobalamin; OH-Cbl, hydroxocobalamin; M, mitochondria; HM, heavy mitochondria; LM, light mitochondria; L, lysosomes; P, plasma membrane–microsomes; S, supernatant cytosol.

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Cell Culture. Monolayer cultures were grown at 37° in a 5% CO_2/95% air atmosphere using Eagle's minimal essential medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 100 μg/ml of kanamycin (Grand Island Biological). Periodic examination of the cell lines for mycoplasmas used broth and agar plates under both aerobic and anaerobic conditions (14). Mycoplasmas were isolated from three cell lines (209, 214, 224) when antibiotics were removed from the culture medium; when mycoplasma growth was suppressed with kanamycin, organisms could not be recovered by the culture methods. Cell extracts were obtained from confluent monolayers harvested with 0.25% trypsin (Grand Island Biological) or, when subcellular fractions were used, with 0.25% pancreatin (Viokase, Grand Island Biological); cell lines had been in culture for 8 to 20 passages.

Cell Extracts. To prepare cell-free extracts for the AdoCbl synthesizing assay, we sonified trypsinized fibroblasts in Ca,Mg-free phosphate-buffered saline three times for 15 sec at 0°, using a Branson sonifier microtip at setting 3. This sonicate was used as the crude enzyme extract. For some experiments the sonicate was centrifuged at 12,000 × *g* for 60 min and a 0–50% saturated $(NH_4)_2SO_4$ precipitate was prepared from the supernatant. This precipitate was redissolved in 0.1 M KPO_4, pH 7.4, and used as the enzyme preparation. Protein determinations were done by the method of Lowry *et al.* (15).

Cell Fractionation. Subcellular fractions from fibroblasts were obtained using modified methods previously published for other tissues (16, 17). After harvesting of cells with Viokase into Ca,Mg-free phosphate-buffered saline, the suspension was centrifuged at 750 × *g* for 10 min and the cell pellet was weighed. The cells were suspended in a hypotonic solution (0.01 M Tris·HCl, pH 7.4, 0.01 M KCl, 0.15 mM $MgCl_2$) at a 1/10 (w/v) ratio and allowed to swell for 45 min at 0° with constant stirring. Sucrose (2.5 M) was then added to a final sucrose concentration of 0.25 M and the cell suspension was immediately forced through a 27 gauge needle 20 times to yield a cell homogenate. The homogenate was centrifuged at 750 × *g* for 10 min to give supernatant 1 and a pellet of unbroken cells (10–25% of the original cells), cell debris, and nuclei. Supernatant 1 was centrifuged at 3300 × *g* for 10 min, yielding a heavy mitochondria–lysosome (HM-L) pellet and supernatant 2. Supernatant 2, after centrifugation for 20 min at 17,000 × *g*, yielded a light mitochondria (LM) pellet and supernatant 3. The latter was centrifuged at 120,000 × *g* (Spinco 40 rotor) for 30 min to give a plasma membrane–microsome (P) pellet and the supernatant cytosol (S). The HM-L pellet was suspended in 200 μl of TMS buffer (0.01 M Tris·HCl, pH 7.4, 0.15 mM $MgCl_2$, 0.25 M sucrose) and layered over 5 ml of 1.2 M sucrose; 100 μl of 0.05 M Tris·HCl, pH 7.4, was layered over the HM-L suspension. This was centrifuged at 53,000 × *g* (Spinco SW 50.1 rotor) for 120 min to give an HM pellet and a supernatant which was further centrifuged at 120,000 × *g* (Spinco 40 rotor) for 30 min to give an L pellet. The HM and LM pellets were combined into a single mitochondria (M) pellet. In some experiments, no attempt was made to obtain separate L and M fractions; the 3300 × *g* centrifugation was omitted and the pellet from the 17,000 × *g* centrifugation was termed an M pellet. The M, L, and P pellets were suspended in 250 μl of 0.1 M KPO_4, pH 7.4, sonicated three times for 15 sec (setting 3) at 0°, and used for enzyme assays. The S fraction was used without further treatment.

Assay for AdoCbl Synthesis. A modification of the method reported by Kerwar *et al.* (8) was used to measure AdoCbl synthesis in the fibroblast extracts and fractions. In the assay OH-[^{57}Co]Cbl was the cobalamin added; this was obtained from quantitative conversion of cyano[^{57}Co]cobalamin (Amersham/Searle, 100–170 Ci/g) (9). Since the first reduction step to cob(II)alamin was accomplished nonenzymatically with dithiothreitol and a hydrogen atmosphere (7), the assay actually measured the final two steps in AdoCbl synthesis, the reduction of cob(II)alamin to cob(I)alamin and the adenosylation of cob(I)alamin to AdoCbl. The assay was carried out anaerobically in Thunberg tubes in a final volume of 1.1 ml containing: 100 μmol of KPO_4, pH 7.4; 0.8 μmol of $MgCl_2$; 0.1 μmol of FAD; 0.05 mmol of dithiothreitol; 1.0 μmol of ATP; OH-[^{57}Co]Cbl in amounts that varied in different experiments; and 100 μl of enzyme preparation [1–2 mg of cell protein as crude extract or $(NH_4)_2SO_4$ precipitate]. The tubes at 0° were evacuated and flushed several times with hydrogen before and after the addition of enzyme preparation. The reaction was started by placing the tubes in a shaking water bath at 37° and was stopped by transferring the tubes to a boiling-water bath for 5 min. The usual incubation time was 30 min. The incubation mixture was transferred quantitatively to another test tube with 0.5 ml of water, held at 75°, and reduced to 0.2–0.25 ml under a stream of nitrogen. The cobalamins were then extracted using ethanol and phenol, separated by thin-layer chromatography, and quantitated by measuring ^{57}Co in a gamma scintillation counter (Packard) as previously described (9). Procedures from the start of incubation until the completion of chromatographic separation were carried out in complete darkness or with minimal red light because of the photolability of AdoCbl. We defined 1 unit of AdoCbl synthesizing activity to be 1 fmol of AdoCbl synthesized per mg of protein in 30 min.

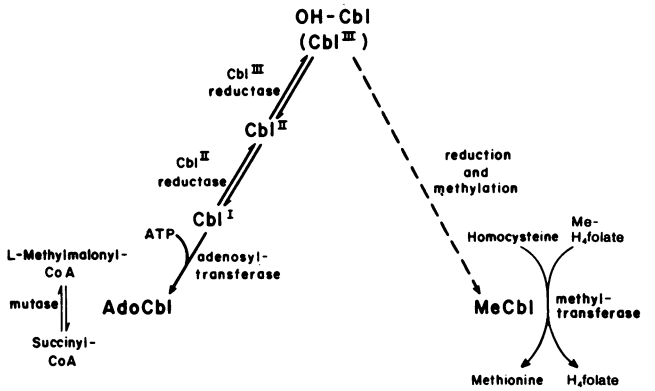

FIG. 1. Intermediary metabolism of cobalamins illustrating the conversion of the cobalamin vitamin, OH-Cbl, to the two cobalamin coenzymes, AdoCbl and MeCbl. Cbl^{III} = cob(III)alamin, etc. The three enzymes known to participate in the synthesis of AdoCbl from OH-Cbl are shown; similar enzymes have yet to be identified in the synthesis of MeCbl. Each cobalamin coenzyme functions as cofactor with a single apoenzyme, AdoCbl with methylmalonyl-CoA mutase and MeCbl with 5-methyltetrahydrofolate-homocysteine methyltransferase. H_4folate, tetrahydrofolate; Me-H_4folate, 5-methyltetrahydrofolate.

Marker Enzyme Assays. Marker enzymes for cell fractions were assayed by standard methods. Succinate dehydrogenase was measured by a spectrophotometric method (18), while the NAD-dependent isocitrate dehydrogenase method (19) was modified to allow fluorometric detection of NADH. These two dehydrogenases were used as mitochondrial

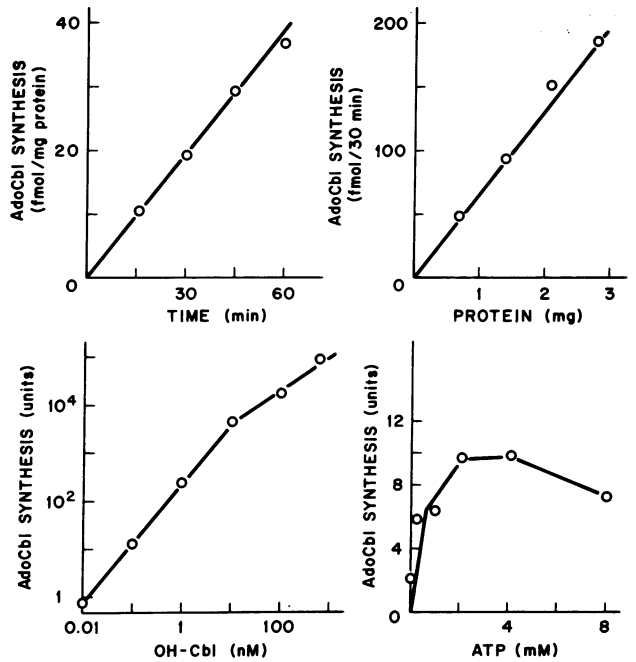

FIG. 2. Radioassay of AdoCbl synthesizing activity in 0–50% $(NH_4)_2SO_4$ precipitates from control fibroblasts. The assay measures the formation of Ado[^{57}Co]Cbl from OH-[^{57}Co]Cbl. Relationships of AdoCbl synthesis to incubation time, protein concentration, initial concentration of each substrate, OH-Cbl and ATP, are illustrated. One unit of AdoCbl synthesizing activity is 1 fmol of AdoCbl synthesized per mg of protein in 30 min. Incubation time was 30 min and the amount of protein in the incubation mixture was 1–2 mg except where either was a variable. OH-Cbl concentration was 0.04 nM when time and ATP concentration were varied and 0.4 nM when protein was varied. ATP concentration was 1 mM except when it was varied. Log scales are used for both axes in the lower left panel, and linear scales in the other three panels.

markers. The lysosomal enzyme, β-galactosidase, was assayed fluorometrically at pH 5.0 using methylumbelliferyl-β-D-galactoside (20); the plasma membrane enzyme, 5′-nucleotidase, by release of inorganic phosphate (21); and the cytosol enzyme, glucose-6-phosphate dehydrogenase, spectrophotometrically (22).

RESULTS

AdoCbl Synthesis in Control Cell Extracts. The requirements for AdoCbl synthesis with crude extracts from control cells as the enzyme source are listed in Table 1. No AdoCbl synthesis was detected when the two substrates, OH-Cbl and ATP, or the chemical reductant, dithiothreitol, were omitted. Removal of FAD or $MgCl_2$ reduced AdoCbl synthesis by 81% and 71%, respectively. Since both cobalamin reductases in bacteria require a flavin cofactor and divalent cations (6, 7), the residual activity we observed after omitting FAD or $MgCl_2$ probably reflects the presence of these cofactors in the fibroblast extract. In the presence of light, AdoCbl, which is photolabile, was not found.

Fig. 2 shows the relationship of AdoCbl synthesis to time, amount of cell protein added, and initial concentrations of the two substrates, OH-Cbl and ATP. All these experiments used the $(NH_4)_2SO_4$ precipitate as enzyme source. Activity was linear with time and protein concentration, findings also demonstrated using crude cell extracts. AdoCbl synthesis was linear with OH-Cbl concentrations from 0.01 to 10 nM,

Table 1. Requirements for AdoCbl synthesis in control fibroblast extracts

Conditions	AdoCbl synthesis (units)*
Complete system in dark	85
— OH-Cbl †	< 4
— ATP	< 4
— Dithiothreitol	< 4
— FAD	16
— $MgCl_2$	25
Complete system in light	< 4

* One unit = 1 fmol of AdoCbl synthesized per mg of protein in 30 min. Limit of detection = 4 units.
† OH-Cbl concentration, 0.4 nM.

and there was a suggestion of saturation above this concentration. AdoCbl synthesis plateaued at ATP concentrations of 2–4 mM and was slightly inhibited at higher concentrations.

Subcellular Localization of AdoCbl Synthesizing Activity. Attempts to obtain pure fractions of cell organelles from fibroblasts were only partially successful. Fig. 3 presents enzyme activities associated with the M, L, P, and S fractions as a percentage of the total activity in those four fractions. The total activity in the fractions plus the activity in the cell debris equaled the activity in the whole cell homogenate for each enzyme. The fractionation was most adequate in obtaining an M fraction with almost all of the isocitrate and succinate dehydrogenase activities and with very little contamination from the cytosol. The M fraction did have significant contamination with lysosomes and plasma membranes as demonstrated by the activity profiles for β-galactosidase and 5′-nucleotidase. The β-galactosidase profile indicated very poor recovery of intact lysosomes in the L fraction. Some lysosomes sedimented with the mitochondria while others were broken, spilling their enzymes into the cytosol.

The profile for AdoCbl synthesis was almost identical to that for isocitrate and succinate dehydrogenase activities. The data indicate that the final two steps of AdoCbl synthesis are intramitochondrial. The virtual absence of AdoCbl synthesizing activity in the S fraction shows that cob(II)alamin reductase and the adenosyltransferase are not active together in either lysosomes or the cytosol. A nuclear fraction was not examined and activity there cannot be excluded.

AdoCbl Synthesis in Mutant Fibroblast Extracts. Fig. 4 shows the data from the seven control lines and the 14 methylmalonicacidemia lines, with the $(NH_4)_2SO_4$ precipitate as enzyme source. One control line was used 10 times; the other six in single experiments. The data from two mutant lines with defective mutase apoenzyme, one line blocked in intact cell accumulation of AdoCbl and MeCbl, and three lines blocked in AdoCbl accumulation only represent single experiments; the remainder of the data from the methylmalonicacidemia lines are mean values from two or three experiments.

The mean value for AdoCbl synthesis in extracts from the seven control lines was 17.8 units (range 5.0–22.8). The control line used ten times had a mean of 9.9 units with a range of 4.2–20.6. Cell lines with a mutase apoenzyme defect or a block in intact cell accumulation of both cobalamin coenzymes had AdoCbl synthesizing activity in broken cell extracts indistinguishable from activity in controls. Those cell lines deficient only in AdoCbl accumulation fell into two

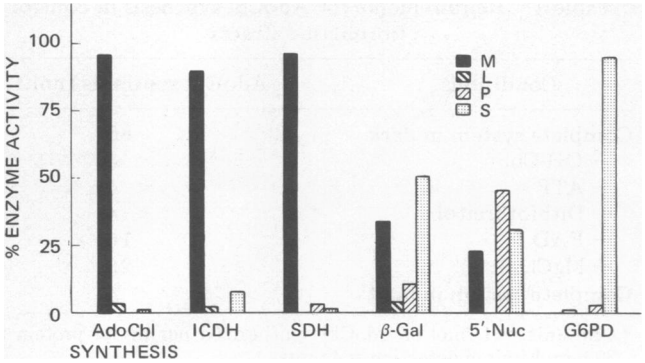

FIG. 3. Percent enzyme activity as a part of the total activity present in three or four subcellular fractions of control fibroblasts. Four fractions were obtained for the AdoCbl synthesis, isocitrate dehydrogenase (ICDH), and β-galactosidase (β-Gal) assays; these were M (mitochondria), L (lysosome), P (plasma membrane-microsome), and S (cytosol) fractions. The other three enzymes (SDH = succinate dehydrogenase, 5'-Nuc = 5'-nucleotidase, G6PD = glucose-6-phosphate dehydrogenase) were assayed in three fractions without the M fraction being further divided into M and L fractions. The activity in these cell fractions plus the activity in a cell debris fraction (including unbroken cells and nuclei) equaled the activity in a whole cell homogenate for each enzyme.

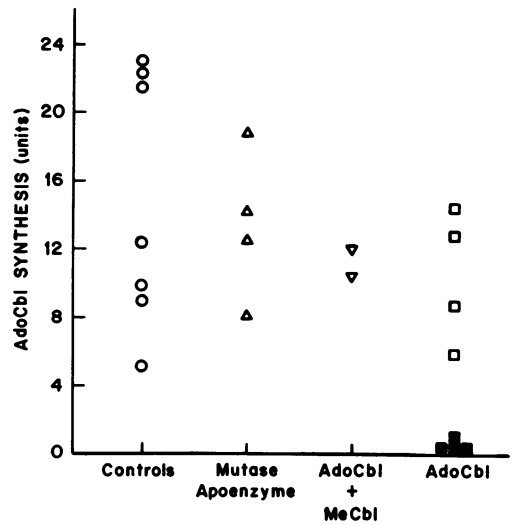

FIG. 4. AdoCbl synthesizing activity in fibroblast extracts from controls and from three groups of patients with methylmalonicacidemia. Cell lines labeled mutase apoenzyme (△) show normal intact cell accumulation of the cobalamin coenzymes, AdoCbl and MeCbl. Intact cells from lines labeled AdoCbl + MeCbl (▽) fail to accumulate both coenzymes. Intact cells from lines labeled AdoCbl fail to accumulate AdoCbl but accumulate MeCbl normally. Four lines in this last group show normal broken cell AdoCbl synthesis (□); four others show deficient synthesis (■). One unit of AdoCbl synthesizing activity is 1 fmol of AdoCbl synthesized per mg of protein in 30 min. The cell extracts were prepared as 0–50% $(NH_4)_2SO_4$ precipitates and the OH-Cbl substrate concentration was 0.04 nM.

groups. Four lines (1f, 214, 221, 245) had normal activities. Line 1f was also examined at a lower OH-Cbl concentration (0.01 nM) and again activity was normal. The remaining four lines had very low (line 215) or undetectable (lines 209, 224, 283) synthesis of AdoCbl; line 215 had 1.0 and 2.1 units of activity in two experiments and undetectable activity (<1.0 unit) in a third. These four lines were also studied at high OH-Cbl concentration (800 nM) and no product was found. With the chromatographic separation and radioassay employed, AdoCbl synthesis at 5% of control activity could have been detected. Crude cell extracts from these four cell lines likewise failed to give evidence of AdoCbl synthesis at both low (0.04 nM) and high (800 nM) concentrations of OH-Cbl. Finally, an 8-fold increase in ATP concentration in the reaction mixture did not result in detectable synthesis of AdoCbl in the four lines.

Mixing of crude extracts from a control cell line and a mutant line deficient in broken cell synthesis gave no evidence of a soluble inhibitor in the mutant extract. These results were found with three mutant lines (209, 215, 224), and data from one such experiment are given in Table 2. No product was detected when only the mutant extract was used. When the two extracts were mixed, more product was formed than predicted by simple addition. One possible explanation for this observation is that cob(II)alamin reductase is rate limiting in the conversion of cob(II)alamin to AdoCbl and that the mutant lines were deficient in cob(I)alamin adenosyltransferase activity but had normal cob(II)alamin reductase activity.

Table 2. AdoCbl synthesis in mixtures of extracts from control cells and mutant cells deficient in AdoCbl synthesis

Control extract (μl)	Mutant* extract (μl)	AdoCbl synthesis† (units)
200	0	168
150	50	164
100	100	103
50	150	70
0	200	< 8

* Cell line 224.

† OH-Cbl concentration, 3.7 nM. One unit = 1 fmol of AdoCbl synthesized per mg of protein in 30 min. Limit of detection = 8 units.

DISCUSSION

Conversion of the vitamin, OH-Cbl, to the coenzyme, AdoCbl, is a three-step enzymatic process. The assay reported in this paper chemically by-passes the first enzyme, cob(III)alamin reductase, and measures the combined activity of the last two, cob(II)alamin reductase and cob(I)alamin adenosyltransferase (7). We cannot exclude some nonenzymatic reduction by dithiothreitol to cob(I)alamin, by-passing the cob(II)alamin reductase, but the requirement for FAD in the system indicates significant activity of cob(II)alamin reductase as well as the adenosyltransferase in the observed synthesis of AdoCbl. We have found that these two enzymes are present in human diploid fibroblasts with a combined specific activity similar to that previously demonstrated in HeLa cells (8). Requirements for FAD, magnesium, and reducing conditions are also very similar to those in HeLa cells.

From cell fractionation studies, we find strong evidence that the part of the AdoCbl synthetic pathway being assayed is intramitochondrial. Work in rat liver (23) and guinea pig ileal mucosa (24) had previously suggested that some of the steps in the conversion of vitamin to coenzyme were occurring within mitochondria. Other biochemical evidence suggested that cob(II)alamin reductase and cob(I)alamin adenosyltransferase would be located together in the cell: first, the

cob(I)alamin species is very unstable under physiologic conditions; and second, attempts to separate cob(II)alamin reductase and the adenosyltransferase in bacteria have been unsuccessful (7). Since methylmalonyl-CoA mutase is, at least in part, a mitochondrial enzyme (25), our findings imply that AdoCbl coenzyme is formed close to the apoprotein with which it will function. Methyltransferase activity, in which MeCbl participates, is located in the cytoplasm (26) but the subcellular site of MeCbl synthesis is not yet known. Should the two coenzymes be synthesized in different organelles, it is possible that completely separate reducing systems exist for the synthesis of each coenzyme.

Earlier definition of inborn errors of cobalamin metabolism using intact cells had recognized two phenotypes, deficient accumulation of AdoCbl, and deficient accumulation of both AdoCbl and MeCbl. The present studies show that the first mutant phenotype is biochemically heterogeneous and that at least two mutant classes are included in this phenotype. We have named those mutants with deficient accumulation of AdoCbl in intact cells but normal broken cell synthesizing activity cbl A mutants; and those with a block in both intact cell accumulation and broken cell synthesis of AdoCbl cbl B mutants. This biochemical heterogeneity implies genetic heterogeneity, and this thesis is supported by data from this laboratory which will be published subsequently (27). Most cbl A and cbl B patients have responded to high dose cobalamin therapy, but one of each class has now been reported who did not respond to vitamin therapy (28). The second intact cell phenotype, failure to accumulate both AdoCbl and MeCbl, defines a third mutant class, cbl C mutants. Fibroblasts from these patients show normal broken cell synthesis of AdoCbl.

Further definition of each mutant class is necessary and further heterogeneity may be found. Since the assay reported here likely combines two enzyme activities, cbl B mutants could have a primary defect at either cob(II)alamin reductase or cob(I)alamin adenosyltransferase. The defect in cbl A mutants could be deficiency of the cob(III)alamin reductase or abnormalities of mitochondrial binding or transport of cobalamins. For cbl C mutants, a defect proximal to the separation of the pathways to AdoCbl and MeCbl must be sought. Since initial cell uptake of free and serum-bound cobalamins is normal in cbl C mutants (29), we must consider such intracellular events as release of cobalamins from lysosomes (23, 30) and binding by cytoplasmic proteins as possible sites for the defect in these cells.

The interesting clinical and biochemical questions posed by these inborn errors of cobalamin metabolism will be answered only by further classification of the sites and processes concerned with intracellular utilization of this vitamin and its coenzymes.

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