Absence of an intracellular cobalamin-binding protein in cultured fibroblasts from patients with defective synthesis of 5'-deoxyadenosylcobalamin and methylcobalamin*

(methylmalonicacidemia/homocystinuria/vitamin B_{12}/coenzyme synthesis)

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ABSTRACT Three distinct classes of human mutations (cbl A, cbl B, and cbl C) cause defective synthesis of cobalamin (Cbl; vitamin B_{12}) coenzymes. Cultured fibroblasts from that unique class (cbl C) deficient in the synthesis of both Cbl coenzymes, 5'-deoxyadenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), were used to explore the underlying defect. We compared the uptake of transcobalamin II (TC II)-bound cyano[^{57}Co]cobalamin (CN-Cbl) by cbl C cells with that of other control and mutant cell lines. Although the cbl C cells initially took up CN-[^{57}Co]Cbl normally, they were unable to retain it. To characterize this "leak" further, cell extracts were prepared following incubation and chromatographed on Sephadex G-150. After incubations of 1–2 hr, most of the CN-[^{57}Co]Cbl accumulated by control cells was still bound to TC II; the remainder was free. Thereafter, an ever-increasing fraction of the labeled Cbl eluted with an intracellular cobalamin-binding protein (ICB); more than 80% of the total was so bound after 76 hr incubations. ICB had an apparent molecular weight similar to that of several Cbl "R" binders (about 120,000), but was distinguished from them by its failure to react with specific anti-"R" binder antiserum. Significantly, no ICB was detected in extracts of three different cbl C lines even after prolonged incubations, whereas its appearance in cbl A, cbl B, and mutase apoenzyme mutants was normal. We propose: that ICB is required for retention of cobalamins by cells; and that cbl C cells "leak" cobalamins and show defective synthesis of Cbl coenzymes because they lack this intracellular binder.

During the past 15 years a picture of the cellular biology and biochemistry of the compound originally designated vitamin B_{12} and now more appropriately referred to by its chemical name, cobalamin (Cbl), has begun to emerge. Based on *in vivo* experiments with radioisotopically labeled Cbl in man (1–3), it has been shown that, once absorbed from the small intestine, the vitamin is transported in blood tightly bound to a specific serum protein, transcobalamin II (TC II). The subsequent delivery of Cbl to hepatocytes (3–7) and other tissue cells (8–13) is mediated by a complicated, still incompletely understood process which is initiated by the binding of the TC II–Cbl complex to specific cell surface receptors. Current evidence favors the belief that the membrane-bound TC II–Cbl complex then enters the cell intact via endocytosis, and is localized transiently in secondary lysosomal vacuoles where proteases degrade TC II, thereby releasing free Cbl into the cytosol (6, 14–16). The vitamin is then metabolized further along two possible paths. It may be converted to methylcobalamin (MeCbl), a required cofactor for the cytoplasmic enzyme, 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13), which catalyzes the methylation of homocysteine to methionine. Alternatively Cbl may enter the mitochondrion and, by a series of enzymatic steps, be converted to the second Cbl coenzyme, 5'-deoxyadenosylcobalamin (AdoCbl). The latter compound is a cofactor for the mitochondrial enzyme methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2), which catalyzes the isomerization of L-methylmalonyl-CoA to succinyl CoA.

Investigations from our laboratory (17–19) and those of others (19–23) have demonstrated that this pathway of cellular cobalamin distribution and coenzyme synthesis is blocked in specific inherited human disorders. At least twenty such patients have been described (24). Although their clinical presentations have varied considerably, biochemical experiments with cultured cells from such patients have allowed us to distinguish three discrete classes of Cbl mutants, designated cbl A, cbl B, and cbl C (25). In cbl A and cbl B mutants, only the synthesis of AdoCbl was blocked in intact cells; MeCbl formation was normal. The third mutant class, cbl C, was characterized by the failure of intact fibroblasts to synthesize both AdoCbl and MeCbl. These cells showed deficient activity of both mutase and methyltransferase (21–23). Complementation tests employing Sendai-virus-mediated heterokaryons confirmed the presence of three complementation groups corresponding to these three chemically defined mutant classes (26).

The present study was undertaken to define the underlying biochemical defect in cbl C mutants. Based on the assumption that the defect in such cells must involve an early step in cellular Cbl metabolism common to the synthesis of both Cbl coenzymes, we examined the uptake of TC II-bound, cobalt-57 labeled cyanocobalamin CN-([^{57}Co]Cbl) by intact monolayers of cultured fibroblasts.

MATERIALS AND METHODS

Cell Lines. Eight skin fibroblast lines were employed: two from controls (lines 87 and 105); three from cbl C mutants (78, 177, 178); and one each from cbl A (1f), cbl B (215), and mutase apoenzyme (184) mutants. Additional information about the source of these particular mutant lines has been presented (26). All cell lines were studied between their 14th and 29th passage in culture, such passages being noted not to affect the ability of cells to take up CN-[^{57}Co]Cbl.

Cell Culture. Fibroblast monolayers were propagated in 75 cm^2 plastic flasks (Falcon) at 37° in a 5% CO_2/95% air atmosphere using Eagle's minimal essential medium supplemented with 10% fetal calf serum (Grand Island Biological), essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Periodic examination of the cell lines for mycoplasmas was carried out as described previously (25); no mycoplasma contamination was found in any of the cell lines used.

Preparation of TC II-Bound CN-[57Co]Cbl. Fresh serum from several healthy volunteers was obtained by venesection, pooled, and frozen in aliquots at −20°. For each experiment a thawed aliquot of serum was incubated with CN-[57Co]Cbl (Amersham/Searle, 90–150 μCi/μg) at a total concentration of 300 pg/ml for 30 min at 37°. Serum was then dialyzed against 0.01 M KPO$_4$ buffer (pH 8.1) for 15–18 hr. More than 95% of the labeled Cbl was uniformly bound to serum under these conditions; 80–90% of the total being bound to TC II. In later experiments dialysis was omitted because it was found to have no effect on serum binding or fibroblast uptake.

Uptake of TC-II-Bound CN-[57Co]Cbl by Monolayers. Monolayers were prepared for uptake studies by pouring off the growth medium, washing three times with a total of 90 ml of phosphate-buffered saline (pH 7.4) at room temperature, and aspirating any retained buffer. Monolayers were then incubated at 37° for intervals of 1–76 hr in 10 ml of medium identical to that employed for cell propagation except that it now contained 10% human serum to which CN-[57Co]Cbl had been bound. The initial medium concentration of labeled Cbl was 25–30 pg/ml.

Incubations were terminated by decanting the medium, washing the monolayer three times with a total of 90 ml of 0.15 M sodium chloride at 4°, and aspirating retained saline. Cells were then removed from the flask by solubilizing them in 1.5 ml of 1% Triton X-100 (New England Nuclear) made up in a 0.15 M sodium chloride, 0.05 M KPO$_4$ buffer (pH 7.4). Alternatively, cells were removed by adding the same volume of Triton-free buffer, harvesting the monolayer by mechanical scraping with a "rubber policeman", sonicating three times (5 sec bursts at 4°) in a Branson sonifier, and centrifuging at 48,000 × g for 10 min at 0°. Of the 1.5 ml of cell extract so obtained, 1.0 ml was used for gel filtration experiments to be described subsequently. The remainder was used for total protein determination by the fluorescamine method of Udenfriend *et al.* (27), or the method of Lowry *et al.* (28), and for 57Co quantitation in an automatic gamma scintillation spectrometer (Packard). Such studies revealed that about 20% of total monolayer radioactivity was not removed by Triton, and that the gel filtration profiles with the two methods were identical.

Gel Filtration with Sephadex G-150. Columns (1.6 × 100 cm) of Sephadex G-150 (Pharmacia), equilibrated with 0.05 M KPO$_4$ buffer (pH 7.4) containing 1% Triton X-100 and 0.15 M NaCl, were used to chromatograph samples (1.0 ml) of 57Co-containing human serum or fibroblast cell extracts. All experiments were carried out at 4° with gel filtration proceeding in the downward direction at a flow rate of 12–15 ml/hr. Individual fractions (1.85 ml) were obtained with an automatic fraction collector and counted as described above.

Sephadex columns were standardized by determining: the void volume (V_0) with Dextran Blue 2000; the total volume (V_t) with CN-[57Co]Cbl; and the elution profiles for TC I and TC II from CN-[57Co]Cbl-saturated human serum. The
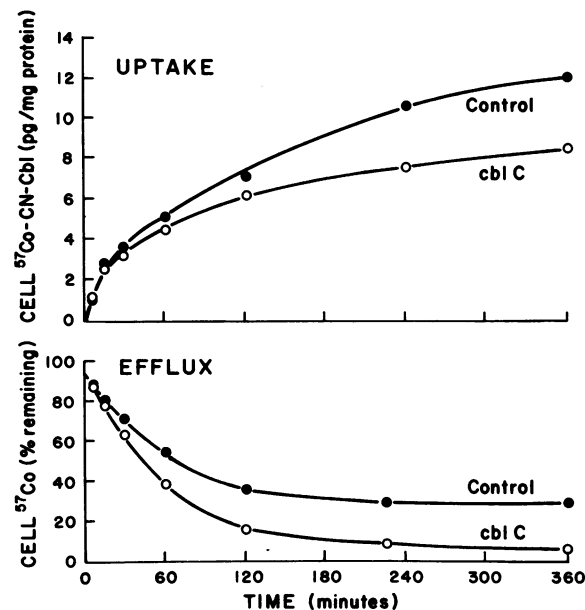

FIG. 1. Upper frame: Uptake of serum-bound CN-[57Co]Cbl (ordinate) plotted against time (abscissa) by intact, confluent cultured fibroblasts from a representative control (105) and cbl C mutant line (78); Lower frame: Efflux of 57 Co from same control and cbl C lines after each had been transferred to fresh, serum-free medium following 6 hr incubations with serum-bound CN-[57Co]Cbl. All studies were carried out at 37° with an initial medium concentration of CN-[57Co]Cbl of 25 pg/ml.

following highly purified proteins were run to facilitate molecular weight estimates: alcohol dehydrogenase (molecular weight M_r—150,000); muconolactone isomerase (M_r—93,000); bovine serum albumin (M_r—67,000); ovalbumin (M_r—45,000); and chymotrypsinogen (M_r—25,000). The partition coefficient, K_{av}, of each protein was determined as follows:

$$K_{av} = V_e - V_0/V_t - V_0$$

where: V_e = elution volume of any particular protein; V_0 = void volume; and V_t = total volume. Triton (1%) was shown not to affect any of these elution profiles.

RESULTS

Uptake of TC-II-bound CN-[57Co]Cbl by fibroblast monolayers

The early time course of uptake of serum-bound CN-[57Co]Cbl by confluent monolayers from representative control and cbl C mutant lines is shown in Fig. 1 (upper frame). In control lines uptake followed a curvilinear plot with accumulation increasing throughout the 360 min interval studied. Virtually identical curves were obtained when monolayers were incubated with CN-[57Co]Cbl bound either to the TC II fraction of human serum or to highly purified human TC II§. In contrast, uptake of free CN-[57Co]Cbl or of labeled vitamin bound to TC I was uniformly less than 10% of that shown in Fig. 1.

Uptake of serum-bound CN-[57Co]Cbl by cbl C mutant lines was indistinguishable from that of controls during the first 30–60 min of incubation but was consistently reduced thereafter. Again, uptake curves for CN-[57Co]Cbl bound to pure TC II gave identical results.

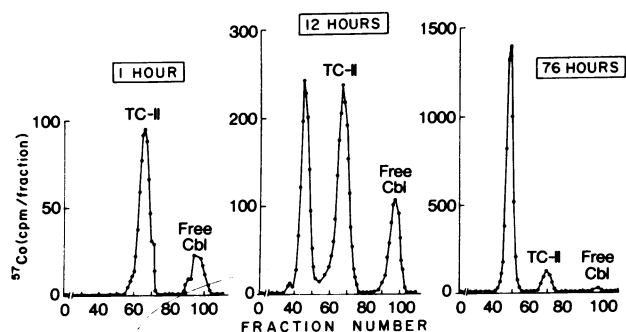§ L. E. Rosenberg, A.-C. Lilljeqvist, and R. H. Allen, unpublished observations.

FIG. 2. Sephadex G-150 elution pattern of ^{57}Co in cell extracts from a representative control line (105). Intact cells were incubated at 37° with serum-bound CN-[^{57}Co]Cbl (25 pg/ml) for 1, 12, or 76 hr; extracts were then prepared with Triton X-100 and chromatographed. Note the ordinate scale differences and the appearance in the 12 and 76 hr samples of a peak of ^{57}Co bound to a macromolecule considerably larger than TC II.

Efflux of ^{57}Co from monolayers

The data in Fig. 1 (upper frame) suggested that the initial binding of TC II-Cbl to cbl C mutant cells is normal, but that the ability of the mutant cells to retain the Cbl accumulated is impaired. We tested this assumption by incubating control and cbl C mutant monolayers with serum-bound CN-[^{57}Co]Cbl for 6 hr, after which the medium was removed, the monolayer was washed exhaustively with cold, isotonic saline, and the cells were then incubated at 37° in serum-free growth medium containing no Cbl. At timed intervals from 5 to 360 min, aliquots of the medium were removed and counted. The fractional loss of ^{57}Co from control and cbl C monolayers is shown in Fig. 1 (lower frame). Although the rate of efflux was very similar in the two cell types ($T_{1/2}$—43 min in control; 41.5 min in cbl C), it is apparent that a considerably greater fraction of ^{57}Co accumulated by the cbl C line was lost to the medium than was noted for control cells. After 6 hr, cbl C cells retained only 5–6% of the ^{57}Co originally taken up, whereas control cells retained 29–30%.

Gel filtration of fibroblast extracts

To characterize further the processes by which CN-[^{57}Co]Cbl is accumulated and retained in normal and mutant cells, we incubated confluent monolayers with TC-II-bound labeled CN-Cbl for various intervals, prepared cell-free extracts as described in *Materials and Methods*, and passed these crude extracts over Sephadex G-150 columns previously standardized with several pure proteins, including TC II. Typical results with extracts of control cells are shown in Fig. 2. When the monolayer was incubated with labeled Cbl for 1 hr (Fig. 2, left) more than 80% of the ^{57}Co co-chromatographed with TC II; the remainder eluted as free Cbl. By 6 hr, a third peak of radioactivity was apparent which chromatographed between the void volume and TC II. After 12 hr incubations (Fig. 2, center), TC II-bound and free Cbl were still readily apparent, but nearly 40% of the total cell ^{57}Co now eluted in the third macromolecular peak. With increasing duration of monolayer incubation, an ever-larger fraction of the total cell ^{57}Co appeared with this peak; greater than 80% of the total was so bound by 76 hr of incubation (Fig. 2, right).

Pronase treatment of this peak resulted in complete disappearance of radioactivity from this region of the chromatogram, thereby demonstrating that the ^{57}Co was bound to a
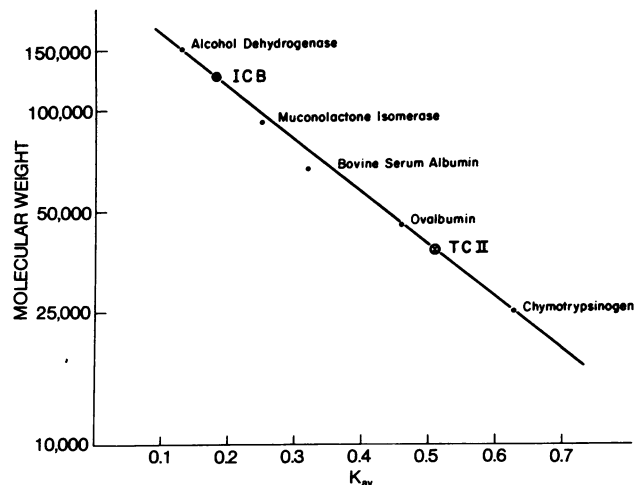

FIG. 3. Estimation of molecular weight of the intracellular cobalamin binder (ICB) found in control cell extracts by means of a Sephadex G-150 column standardized with several homogeneous proteins, including TC II. K_{av} is defined in *Materials and Methods*.

protein. Since the peak was observed in extracts prepared by scraping and sonication with an identical time course and in identical fractional amounts to those observed with Triton-solubilized extracts, it is clear that this protein peak is not a detergent artifact. The delay in its appearance, and our failure to detect it in serum-free culture medium after either short (6 hr) or long (76 hr) incubations, suggested that it was an intracellular protein. We, therefore, referred to it as intracellular cobalamin binding protein, abbreviated ICB.

Differentiation of ICB from known "R" binders

As shown in Fig. 3, the elution profile of ICB indicated an apparent molecular weight of 120,000, a value very similar to that reported for several Cbl-binding proteins found in serum, granulocytes, saliva, and milk which cross react immunologically and are collectively referred to as "R" binders (29). To test the thesis that ICB was another "R" binder, we conducted immunochemical experiments using specific, rabbit anti-human "R" binder antiserum[¶]. First, we demonstrated that the expected antigen–antibody reaction would take place in the presence of fibroblast extracts and would be detectable by gel filtration. When ^{57}Co-labeled "R" binder from human saliva was added to a control fibroblast extract, it eluted from Sephadex with an expected apparent molecular weight of about 120,000 (Fig. 4A, solid line). When anti-"R" binder antiserum was also added before gel filtration, all radioactivity shifted to the void volume, as anticipated for the appearance of a labeled antigen–antibody complex (Fig. 4A, dashed line). Similar experiments were then carried out with extracts of control fibroblasts incubated for 76 hr with CN-[^{57}Co]Cbl (Fig. 4B). It is apparent that the addition of a large excess of anti-"R" binder antiserum (dashed line) shifted none of the radioactive material in the ICB peak to the void volume. Such experiments were also performed with anti-TC II antiserum to exclude the unlikely possibility that ICB represented a TC II aggregate. Again, no immunochemical reactivity between the antiserum and ICB was observed.
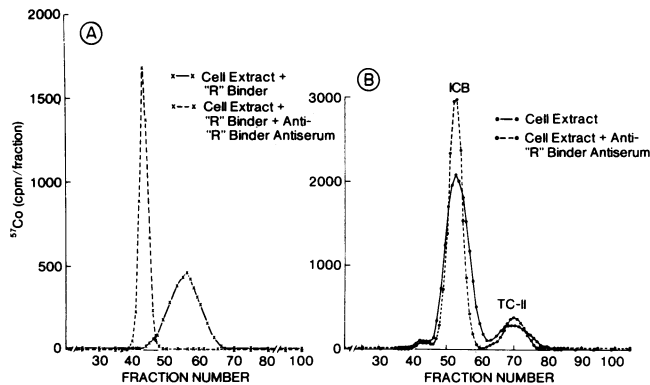
FIG. 4.   (A) Effect of addition of specific, rabbit anti-"R" binder antiserum (dashed line) on Sephadex G-150 elution profile of ⁵⁷Co-labeled, crude salivary "R" binder (solid line) added to a control cell extract. Note the shift of all radioactivity to the void volume following addition of 10 μl of antiserum. (B) Failure of anti-"R" binder antiserum (dashed line) to alter elution profile of ICB from cell extract prepared from a control monolayer incubated for 76 hr with TC II-bound CN-[⁵⁷Co]Cbl (25 pg/ml). Addition of 10 and 100 μl of antiserum gave identical results.

Absence of ICB in cbl C extracts

No detectable ICB peak was observed when cell extracts from each of three cbl C mutant lines were chromatographed on Sephadex (Fig. 5). Neither after short incubations (6 hr) nor long ones (76 hr) did we observe detectable counts above background in this region. The TC II and free Cbl peaks in cbl C extracts were similar to those found in control extracts, and no new peak was found to replace ICB in the mutant extracts. The specificity of this abnormality in cbl C lines was demonstrated by chromatographing extracts of other lines with deficient methylmalonyl-CoA mutase activity: a mutase apoenzyme mutant; a cbl A mutant; and a cbl B mutant. As shown in Table 1, the fraction of total ⁵⁷Co in each of these cell extracts which was present as ICB was very similar to that in control cells, after both short and long incubations.

Table 1.   Intracellular cobalamin binding protein (ICB) in extracts of control and mutant cell lines

Cell phenotype†	% of total cellular ⁵⁷Co as ICB*	
	6 hr incubation	76 hr incubation
Control (105)	30	85
Mutase apoenzyme mutant (184)	21	83
cbl A mutant (1f)	28	87
cbl B mutant (215)	20	81
cbl C mutant (78)	0	0

* Confluent monolayers were incubated for 6 and 76 hr in growth medium containing TC-II-bound CN-[⁵⁷Co]Cbl (25 pg/ml). Thereafter, cell extracts were prepared as described in *Materials and Methods* and chromatographed on Sephadex G-150. The total radioactivity recovered from the chromatogram was summed and that fraction found in the ICB peak was calculated.

† Each line is identified according to phenotype and, in parentheses, laboratory number.

DISCUSSION

Our findings demonstrate that, as in a variety of other mammalian cells, TC II facilitates the uptake of cyanocobalamin by cultured diploid human fibroblasts. Our data offer no new insights into the mechanisms by which the TC II–Cbl complex binds to the cell membrane or enters the intracellular space. They do show that, with increasing duration of incubation, there is a progressive shift of the labeled Cbl from TC II to a protein with an apparent molecular weight of 120,000. Such a cellular cobalamin-binding protein has been observed previously in extracts of ascites tumor cells (13) and rat liver (14) after exposure to labeled CN-Cbl, but little is known about its site or mechanism of synthesis, its subcellular localization, its function, or the nature of the cobalamin species which bind to it.

We wish to emphasize several points about the cellular binding protein identified in our studies with normal cells.
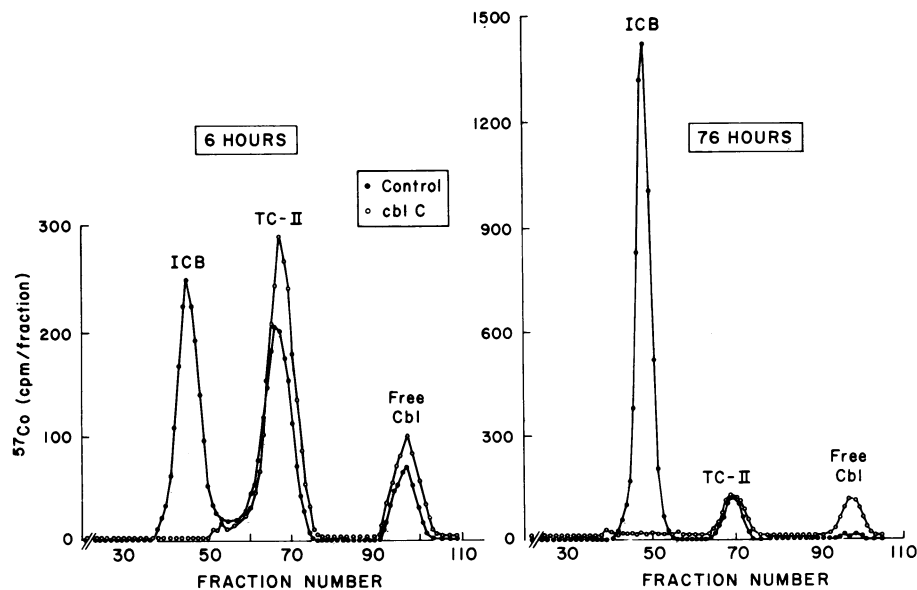

FIG. 5.   Absence of ICB peak in extracts of a representative cbl C mutant line (O) after intact cells were incubated with CN-[⁵⁷Co]Cbl (25 pg/ml) for 6 hr (left frame) or 76 hr (right frame). Identical results were obtained in repeated studies of all three cbl C lines (78, 177, 178). Data from a control line (105; ●) are shown for comparison.

First, its delayed appearance and its preliminary subcellular localization to the soluble fraction and/or mitochondria[||] indicate that it is an *intracellular* protein, which we designated ICB for intracellular cobalamin binder. Second, our inability to detect any *in vitro* binding of free CN-[57Co]Cbl or free OH-[57Co]Cbl to ICB in cell extracts[||], as contrasted with its major appearance following incubations of intact cells with TC-II-bound Cbl, suggests that its synthesis or its ability to bind Cbl may require other, still unidentified metabolic events. Third, its failure to react immunochemically with anti-"R" binder antiserum differentiates it from that class of Cbl binder. Fourth, the fact that each of the major intracellular Cbl species (OH-Cbl; MeCbl and AdoCbl) was recovered from the ICB peak after cells were incubated for 72 hr[||] raises the possibility that ICB is involved in some way with Cbl interconversion and coenzyme synthesis.

The most dramatic, and potentially most significant, observation relevant to ICB is its absence in three cbl C mutant lines. Since the endogenous total Cbl content of such mutant cells is only 15–25% of that of controls[||], our failure to detect ICB cannot be ascribed to saturation with unlabeled Cbl. It could also be argued that the appearance of ICB reflects, in some way, mutase activity or Cbl coenzyme synthesis, but this hypothesis is hardly tenable in the face of normal ICB appearance in cbl A and cbl B mutant lines or in those with a mutase apoenzyme defect. Rather, we wish to propose that cbl C mutants are deficient in AdoCbl and MeCbl synthesis *because* they lack ICB activity. Such lack could result from primary deficiency of ICB or from an abnormality of a metabolic step required for Cbl binding to ICB. We propose further that this deficiency of ICB activity results in the inability of cbl C mutant cells to retain the Cbls which they accumulate, thereby leading to the exaggerated efflux of CN-[57Co]Cbl observed in our experiments and to the reduced endogenous Cbl content noted in many tissues of these patients (20–23).

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