

# Cobalamin Binding and Cobalamin-Dependent Enzyme Activity in Normal and Mutant Human Fibroblasts

IRA MELLMAN, HUNTINGTON F. WILLARD, and LEON E. ROSENBERG,  
*Department of Human Genetics, Yale University School of Medicine,  
New Haven, Connecticut 06510*

**ABSTRACT** We have studied the intracellular binding of radioactive cobalamin by normal cultured human fibroblasts grown in medium containing [<sup>57</sup>Co]-cobalamin. We have also assessed the significance of defects in this binding activity exhibited by two classes of human mutants (*cbl C* and *cbl D*) each characterized by pleiotropic deficiencies in the accumulation and retention of cobalamin, in the synthesis of cobalamin coenzymes, and accordingly, in the holoenzyme activities of both cobalamin-dependent enzymes, 5-methyltetrahydrofolate:homocysteine methyltransferase and methylmalonyl-CoA mutase. Based on the coincidence of [<sup>57</sup>Co]cobalamin binding and cobalamin-dependent enzyme activities after Sephadex G-150 chromatography and polyacrylamide gel electrophoresis, we conclude that, as in rat liver, the intracellular binding of labeled cobalamin by normal fibroblasts reflects the attachment of the vitamin to the cobalamin-dependent methyltransferase and mutase. Whereas *cbl C* cells are completely deficient in the binding of [<sup>57</sup>Co]cobalamin to either enzyme, fibroblasts which bear the phenotypically similar but genetically distinct *cbl D* mutation retain some binding activity, and accordingly, have higher holomethyltransferase and holomutase activities than do *cbl C* cells. The defect in [<sup>57</sup>Co]cobalamin binding exhibited by both *cbl C* and *cbl D* fibroblasts is almost certainly not a result of mutations which affect the methyltransferase or mutase apoenzymes, since the electrophoretic mobilities and the affinities of these enzymes for their respective cobalamin coenzymes are indistinguishable from those in control

cell extracts. These results suggest that both the *cbl C* and *cbl D* mutations affect some enzymatic step(s) which converts newly taken up cobalamin to a form capable of being bound by the two cobalamin-dependent enzymes.

## INTRODUCTION

Detailed study of cultured fibroblasts from patients with inherited methylmalonic acidemia has shown that mutations affecting human cobalamin (Cbl or vitamin B<sub>12</sub>)<sup>1</sup> metabolism constitute at least four complementation groups, referred to as *cbl A*, *cbl B*, *cbl C*, and *cbl D* (1-3). Individuals expressing either the *cbl C* or *cbl D* mutations can be differentiated from the other mutant phenotypes in that they exhibit homocystinuria in addition to elevated concentrations of serum and urine methylmalonic acid. This finding indicates that the *cbl C* and *cbl D* mutations affect the function of both Cbl-dependent enzymes: methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carboxylmutase, EC 5.4.99.2) catalyzing the isomerization of methylmalonyl-CoA to succinyl-CoA, and 5-methyltetrahydrofolate:homocysteine methyltransferase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase, EC 2.1.1.13) which mediates the methylation of homocysteine to form methionine (4).

Previous investigations have shown that intact *cbl C* and *cbl D* fibroblasts are deficient in Cbl accumulation, in the synthesis of the two Cbl coenzymes, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl), and accordingly in the holoenzyme activities of their respective apoenzymes, mutase and methyltransferase (2, 5-8). In 1975, a possible explanation for this pleiotropic phenotype was suggested by our observation that *cbl C* cells were unable to bind Cbl to an intra-

---

This work was presented in part at the meeting of the American Society of Human Genetics, San Diego, Calif., October 1977.

Dr. Mellman and Dr. Willard are recipients of traineeships from the National Institutes of Health (T01-GM 02299). Dr. Mellman's present address is Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021.

Received for publication 18 May 1978 and in revised form 23 June 1978.

---

<sup>1</sup>Abbreviations used in this paper: AdoCbl, adenosylcobalamin; Cbl, cobalamin or vitamin B<sub>12</sub>; CN-Cbl, cyanocobalamin; MeCbl, methylcobalamin; MeFH<sub>4</sub>, N<sup>5</sup>-methyltetrahydrofolate; TC II, transcobalamin II.

cellular protein(s) (9). When crude extracts of control fibroblasts grown in medium that contained cyano- $^{57}\text{Co}$ ]Cbl (CN- $^{57}\text{Co}$ ]Cbl) were chromatographed on Sephadex G-150, almost 90% of the label was found associated with a single protein peak of an apparent mol wt of 120,000. On the other hand, *cbl C* cells treated in the same manner showed literally no such binding, although their initial uptake of CN- $^{57}\text{Co}$ ]Cbl from the medium appeared to be normal. Therefore, we postulated that a distinct Cbl-binding protein species, whose function might be to accumulate and retain intracellular Cbl for coenzyme synthesis, was deficient in *cbl C* fibroblasts.

More recently, however, we and others have obtained evidence suggesting that in rat (and rabbit) liver, virtually all intracellular Cbl is bound to the Cbl-dependent enzymes, mutase and methyltransferase (10, 11). As a result of these findings, we have initiated a series of investigations designed to better understand the nature of intracellular Cbl binding in cultured human fibroblasts and to assess the significance of its absence in mutant cells.

The first of these reports is presented here. We have found that, as in rat liver, the intracellular binding of  $^{57}\text{Co}$ ]Cbl in normal cultured fibroblasts can be accounted for by its attachment to the two Cbl-dependent apoenzymes; that cells expressing the recently identified *cbl D* mutation (3) are phenotypically similar to *cbl C* cells with respect to a reduced amount of intracellular  $^{57}\text{Co}$ ]Cbl binding; and finally, that neither the *cbl C* nor the *cbl D* mutation appears to affect a gene specifying a protein subunit common to both mutase and methyltransferase.

## METHODS

**Materials.** L-Homocysteine thiolactone hydrochloride, S-adenosylmethionine iodide, and AdoCbl were obtained from Calbiochem, San Diego, Calif.; DL-N<sup>5</sup>-methyltetrahydrofolate (MeFH<sub>4</sub>), MeCbl, and cyanocobalamin (CN-Cbl) were purchased from Sigma Chemical Co., St. Louis, Mo. Before use, MeFH<sub>4</sub> was converted to the sodium salt by incubating in buffer that contains 0.03 M NaHCO<sub>3</sub>, 0.1 M Na<sub>2</sub>HPO<sub>4</sub> and 0.1 M β-mercaptoethanol for 30 min at 0°C.

[1-<sup>14</sup>C]Propionate (50 mCi/mmol) and DL-[methyl-<sup>14</sup>C]-methylmalonyl-CoA (45–55 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. CN- $^{57}\text{Co}$ ]Cbl (100–200 Ci/mmol) and DL-N<sup>5</sup>-[methyl-<sup>14</sup>C]MeFH<sub>4</sub> (monoglutamate, 53–56 mCi/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. For use in enzyme assays, [<sup>14</sup>C]MeFH<sub>4</sub> was further purified by chromatography in the dark on a column of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N. J.) (0.6 cm × 110 cm) equilibrated in 0.1 M potassium phosphate (pH 7.4) that contained 0.02 M β-mercaptoethanol. MeFH<sub>4</sub> was sufficiently retarded on this column (12) to allow its separation from radioactive contaminants in the commercial preparation. 1-ml fractions were collected and the OD at 280 nm was monitored. A major peak of absorbance, eluted after approximately 100 ml had been collected, contained >95% of the radioactivity. These fractions

were pooled and concentrated in the dark by lyophilization. MeFH<sub>4</sub> was identified by its ultra-violet-visible absorbance spectra in 0.1 M potassium phosphate (pH 7) and in 0.1 M HCl (pH 1) (13). Purified [<sup>14</sup>C]MeFH<sub>4</sub>, diluted to 2 mM with cold MeFH<sub>4</sub> and stored under argon at -20°C in the presence of 0.1 M β-mercaptoethanol, was stable for several months. This procedure reduced the background and thus increased the sensitivity of the methyltransferase assay (*vide infra*) 5- to 10-fold.

**Cells and cell culture.** Human skin fibroblasts were routinely maintained in 32-ounce glass bottles in Eagle's minimal essential medium supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.), and 100 μg/ml kanamycin (Grand Island Biological Co.). For experiments, cells were plated at a 1:4 split ratio into 150-cm<sup>2</sup> tissue culture flasks or 490-cm<sup>2</sup> plastic roller bottles and used 4–5 days later. To facilitate vitamin uptake, cells were labeled with  $^{57}\text{Co}$ ]Cbl bound to transcobalamin II (TC II) as previously described (9). 24 h after plating, monolayers were refed with regular growth medium that contained 10% human serum (as a TC II source) which had previously been incubated with CN- $^{57}\text{Co}$ ]Cbl (0.5 ng/ml serum) for 30 min at 37°C. Cells were maintained in this medium 72 h before harvest with 0.25% trypsin/EDTA.

Four control, four *cbl C*, and two *cbl D* cell lines were employed in these studies. The mutant cell lines were derived from six patients with methylmalonic acidemia and homocystinuria and were characterized by complementation analysis (3).<sup>2</sup> Cells had been in culture between 12 and 29 passages before use.

**Assay of MeFH<sub>4</sub>:homocysteine methyltransferase activity.** Methyltransferase activity in cell-free extracts was determined by measuring the production of [<sup>14</sup>C]methionine from [<sup>14</sup>C]MeFH<sub>4</sub> according to a modification of the procedure of Mudd et al. (5). Cells were harvested 4 days after plating with 0.25% trypsin/EDTA, centrifuged at 700 g for 2.5 min, and washed twice with calcium- and magnesium-free Dulbecco's phosphate-buffered saline. Pellets were sonicated in 0.1 M potassium phosphate (pH 7.4) at 0°C using four 15-s bursts at 15-s intervals. Sonicates were centrifuged 10 min at 48,000 g and the supernate used for assay. In a final volume of 0.2 ml, the reaction mixture contained: 20 μmol potassium phosphate (pH 7.4), 50 nmol S-adenosylmethionine, 25 μmol β-mercaptoethanol, 50 nmol L-homocysteine (prepared daily from the thiolactone), 10 nmol MeCbl, 50–100 nmol [<sup>14</sup>C]-MeFH<sub>4</sub> (final sp act ≈ 2000 cpm/nmol), and 50–300 μg cell protein. Holoenzyme activity is defined as that activity which exists when the assay is conducted without added MeCbl; inclusion of MeCbl measures total (holoenzyme plus apoenzyme) activity. All procedures involving alkylcobalamins were carried out in the dark. Tubes were flushed with nitrogen and incubated at 37°C for 90 min. The reaction was terminated by the addition of 0.8 ml cold distilled water. [<sup>14</sup>C]Methionine was recovered by applying the reaction mixture to a Pasteur pipet column of Bio-Rad AG1-X8 resin (Bio-Rad Laboratories, Richmond, Calif.) (100–200 mesh, chloride form) and washing the column with an additional 1 ml of water. The 2 ml of effluent was pooled, 15 ml Aquasol (New England Nuclear) was added, and radioactivity assayed in a Packard liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Methyltransferase activity is expressed as picomoles methionine formed per minute per milligram protein. Protein was measured by the method of Lowry et al. (14).

<sup>2</sup> Cell lines representing both of these complementation groups are available from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N. J.

**Methylmalonyl-CoA mutase assay.** Mutase activity in cell-free extracts was determined by the production of [ $^{14}\text{C}$ ]succinate from [ $^{14}\text{C}$ ]methylmalonyl-CoA as described previously (15). Total enzyme activity (holoenzyme plus apoenzyme) was measured by including  $5\ \mu\text{M}$  AdoCbl in the reaction mixture; holoenzyme activity was that which existed without added cofactor.

**Intact cell assays.** The activities of both mutase and methyltransferase were estimated in intact cells by measuring the activities of the overall metabolic pathways in which they function. Thus, the incorporation of  $^{14}\text{C}$  from [ $^{14}\text{C}$ ]propionate into acid-precipitable material (16) was taken as an indication of intact cell mutase activity (see above and Fig. 2). The conversion of [ $^{14}\text{C}$ ]methylmalonyl-CoA to [ $^{14}\text{C}$ ]succinyl-CoA becomes rate limiting when there is a relative deficiency of holomutase activity.<sup>3</sup>

Similarly, the incorporation of  $^{14}\text{C}$  from [ $^{14}\text{C}$ ]MeFH<sub>4</sub> (via endogenous [ $^{14}\text{C}$ ]methionine synthesis) was taken as an estimate of methyltransferase activity. A modification of the method of Hoffman and Erbe (17) was employed. Briefly,  $8 \times 10^6$  cells were plated in 60-mm tissue culture dishes and 48 h later, re-fed with 2 ml of methionine- and folic acid-free minimal essential medium that contained 10% dialyzed fetal calf serum, 0.2 mM L-homocysteine thiolactone, 50  $\mu\text{g}/\text{ml}$  sodium ascorbate, and 8–9  $\mu\text{M}$  [ $^{14}\text{C}$ ]MeFH<sub>4</sub>. After 18 h at 37°C, this medium was removed and the monolayers washed twice with calcium- and magnesium-free Dulbecco's phosphate-buffered saline. Cold 5% TCA was then added

and the plates incubated for 15 min at 4°C. After four additional washes with 5% TCA, the precipitate was dissolved in 0.2 M NaOH and assayed for protein (14) and radioactivity (16). "Nonspecific"  $^{14}\text{C}$  incorporation, estimated by incubation of parallel samples in medium containing 0.1 mM methionine in the absence of homocysteine, was routinely subtracted.

**Gel filtration.** Columns of Sephadex G-150 (1.6  $\times$  100 cm), equilibrated in 0.05 M potassium phosphate (pH 7.4) that contains 0.15 M NaCl, were standardized and run as described (9, 10). Washed cell pellets were resuspended in 1–2 ml of column buffer, sonicated, and centrifuged at 48,000 g for 20 min before being applied to the column. Typically, over 75% of the cellular [ $^{57}\text{Co}$ ]Cbl was solubilized by this procedure.

**Polyacrylamide gel electrophoresis.** Both tube and slab gels (7 cm in length; 4% in acrylamide) were run without stacking gels according to the method of Davis (18). Cell extracts were prepared by sonicating washed pellets in 0.02 M Tris/HCl (pH 7.4) at 0°C and centrifuging the sonicate at 105,000 g for 1 h. Gels were frozen, sliced into 1 mm-fractions, and mutase or methyltransferase activity determined by incubating slices in the appropriate reaction mixture in the presence of AdoCbl or MeCbl.  $^{57}\text{Co}$  was measured in each slice with a Packard Auto-Gamma spectrometer (Packard Instrument Co.).

## RESULTS

**Identification of [ $^{57}\text{Co}$ ]Cbl-binding proteins in cultured fibroblasts.** To determine the relationship between Cbl binding and Cbl-dependent enzyme

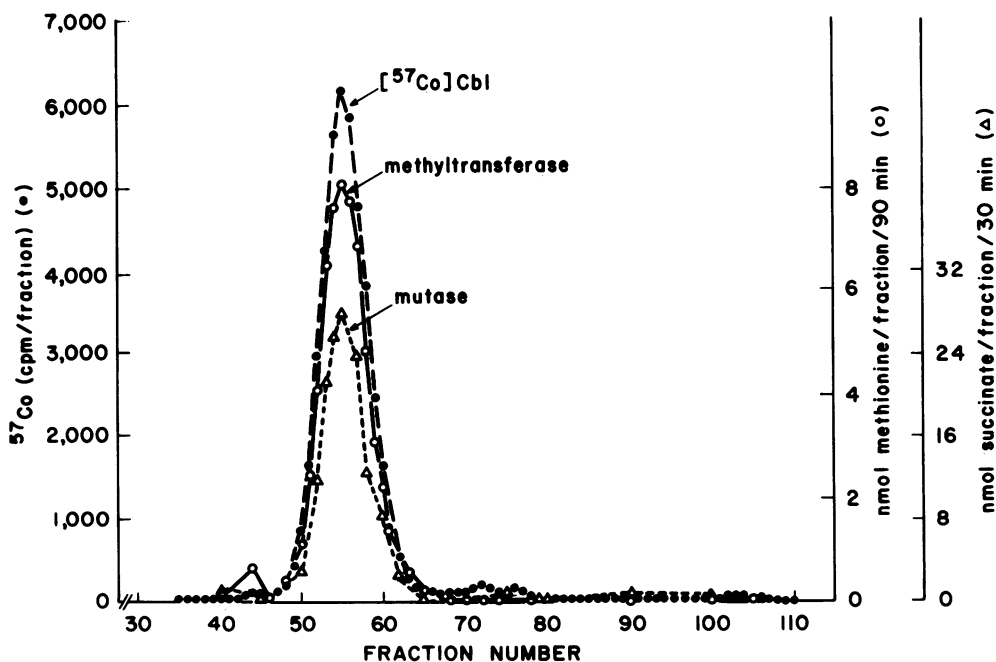


FIGURE 1 Sephadex G-150 gel-filtration profiles of [ $^{57}\text{Co}$ ]Cbl binding ( $\bullet$ --- $\bullet$ ), MeFH<sub>4</sub>: homocysteine methyltransferase activity ( $\circ$ — $\circ$ ), and methylmalonyl-CoA mutase activity ( $\Delta$ — $\Delta$ ) from extracts of control cultured human fibroblasts. Methyltransferase activity is expressed as nanomoles [ $^{14}\text{C}$ ]methionine formed per fraction per 90 min; mutase activity is expressed as nanomoles [ $^{14}\text{C}$ ]succinate formed per fraction per 30 min. The major [ $^{57}\text{Co}$ ]Cbl-binding peak chromatographs with an apparent mol wt of 120,000 (9, 10).

activity in normal cultured human fibroblasts, extracts were prepared from cells grown in medium that contained TC II-bound CN-[ $^{57}\text{Co}$ ]Cbl (see Methods), chromatographed on Sephadex G-150, and column fractions assayed for  $^{57}\text{Co}$ , mutase activity, and methyltransferase activity. The results of such an experiment are presented in Fig. 1. Almost 90% of the intracellular [ $^{57}\text{Co}$ ]Cbl was found associated with a single high mol wt ( $\approx 120,000$ ) peak; only small amounts were bound to TC II or present as free (not protein bound) Cbl. Moreover, the data clearly show that both mutase and methyltransferase activities co-chromatographed with the major [ $^{57}\text{Co}$ ]Cbl peak, thus suggesting that Cbl-binding activity in these cells was due in large part to the attachment of [ $^{57}\text{Co}$ ]Cbl to the two Cbl-dependent apoenzymes.

As shown in Fig. 2, co-chromatography of binding and enzyme activities was also observed after polyacrylamide gel electrophoresis. In this case, however, the binding peak was resolved into two components. The more anodal of these accounted for almost 80% of the  $^{57}\text{Co}$  applied to the gel and migrated with methyltransferase activity. The minor [ $^{57}\text{Co}$ ]Cbl-binding peak was found to be associated at least in part with mutase activity. Although TC II-bound [ $^{57}\text{Co}$ ]Cbl ran in this region (data not shown), its actual contribution to the smaller peak was found to be insignificant. When TC II-[ $^{57}\text{Co}$ ]Cbl was removed by Sephadex G-150 chromatography before electrophoresis, the [ $^{57}\text{Co}$ ]Cbl-binding profile was indistinguishable from that shown in Fig. 2.

These results are in close agreement with those obtained from our more extensive investigation of Cbl binding in rat liver which demonstrated that the correspondence of [ $^{57}\text{Co}$ ]Cbl binding and Cbl-dependent enzyme activity was maintained after subcellular fractionation and DEAE-cellulose chromatography as well as gel filtration and electrophoresis (10). Therefore, we conclude that in cultured human fibroblasts the vast majority of intracellular [ $^{57}\text{Co}$ ]Cbl binding can be accounted for by the attachment of [ $^{57}\text{Co}$ ]Cbl to the two Cbl-dependent apoenzymes, methyltransferase and (to a lesser extent) mutase. Thus, the absence of intracellular [ $^{57}\text{Co}$ ]Cbl binding in *cbl C* cells is not likely to be explained by a mutation affecting a discrete Cbl-binding protein, as was previously suggested (9).

**[ $^{57}\text{Co}$ ]Cbl binding in mutant cells.** In view of these results, we re-examined [ $^{57}\text{Co}$ ]Cbl binding in mutant and control fibroblasts using both Sephadex G-150 chromatography and gel electrophoresis. In particular, it was of interest to learn whether *cbl D* cells, which are similar to *cbl C* cells in their inability to synthesize either Cbl coenzyme, are likewise deficient in the intracellular binding of [ $^{57}\text{Co}$ ]Cbl. Fig. 3 contains the results of these experiments. As observed previously (9), the Sephadex G-150 profile of extracts from *cbl C* fibroblasts grown in TC II-[ $^{57}\text{Co}$ ]Cbl indicated that these cells did not bind detectable amounts of radioactivity to the Cbl-dependent apoenzymes. Similarly, no such binding was observed after the *cbl C* extracts were subjected to electrophoresis. The small amount of radioactivity present on the *cbl C* gel was probably a

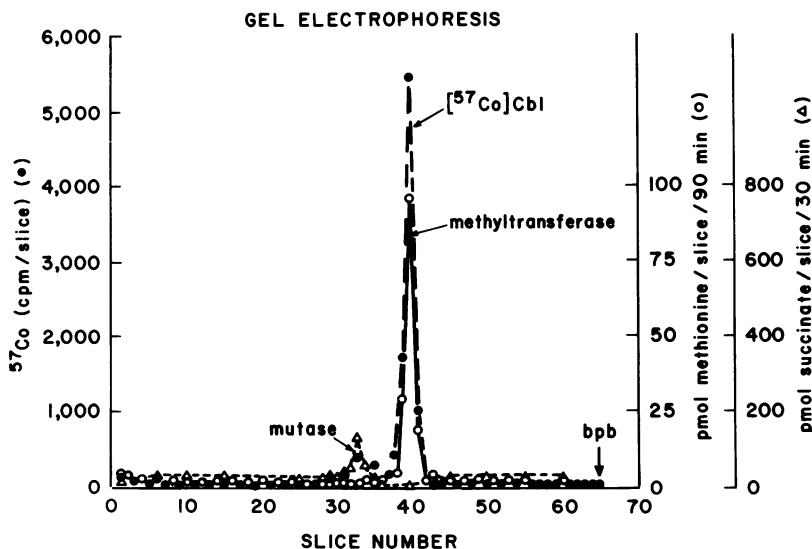


FIGURE 2 Profile of [ $^{57}\text{Co}$ ]Cbl binding (● --- ●), MeFH<sub>4</sub>:homocysteine methyltransferase activity (○ — ○), and methylmalonyl-CoA mutase activity (Δ --- Δ) after polyacrylamide-gel electrophoresis of labeled control fibroblast extracts. The migration of the marker bromophenol blue (bpb) is indicated.

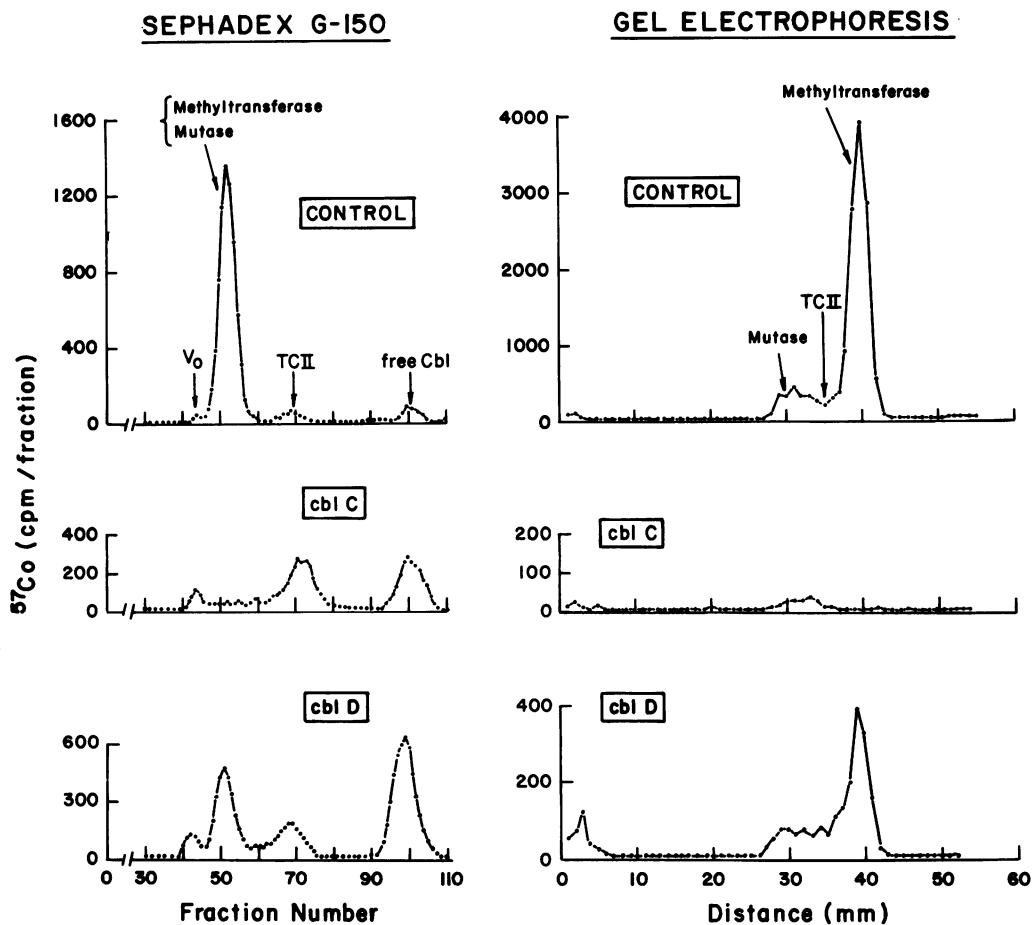


FIGURE 3 Comparison of typical [ $^{57}\text{Co}$ ]Cbl-binding profiles of control, *cbl C*, and *cbl D* fibroblasts after Sephadex G-150 gel filtration and polyacrylamide-gel electrophoresis. Cells were labeled and extracts prepared as described in Methods. Identical amounts of cell protein were used for each experiment.

result of internalized TC II- $^{57}\text{Co}$ ]Cbl (which runs as a relatively wide band on this system), because free [ $^{57}\text{Co}$ ]Cbl does not enter the gel.

The Sephadex G-150 profile of extracts prepared from [ $^{57}\text{Co}$ ]Cbl-labeled *cbl D* fibroblasts, however, showed a significant amount of [ $^{57}\text{Co}$ ]Cbl associated with the high molecular weight peak (Fig. 3), although clearly less than that bound in controls (identical amounts of control and *cbl D* cell protein were applied to the columns). Gel electrophoresis of the *cbl D* extracts indicated that most of this residual binding was due to attachment of [ $^{57}\text{Co}$ ]Cbl to the more rapidly migrating Cbl-binding protein, the methyltransferase. Moreover, this binding activity was of an electrophoretic mobility identical to that from controls. Thus, whereas the *cbl D* mutation results in a clinical and cellular phenotype otherwise similar to that characteristic of the *cbl C*, *cbl D* fibroblasts are distinguished by their ability to

bind a reduced but intermediate amount of [ $^{57}\text{Co}$ ]Cbl to the Cbl-dependent apoenzymes.

*Cbl-dependent enzyme activity in mutant cells.* To determine whether the residual [ $^{57}\text{Co}$ ]Cbl binding in *cbl D* fibroblasts was reflected in increased levels of mutase and methyltransferase holoenzyme relative to those found in *cbl C* cells, Cbl-dependent enzyme activities were measured in both mutants and controls. First, endogenous holoenzyme activity was estimated in intact cells by incubating fibroblasts in medium that contained either [ $^{14}\text{C}$ ]propionate or [ $^{14}\text{C}$ ]MeFH $_4$ . The fixation of [ $^{14}\text{C}$ ]propionate (via its conversion to [ $^{14}\text{C}$ ]methylmalonyl-CoA and ultimately to [ $^{14}\text{C}$ ]amino acids) was taken as a measure of mutase activity (16) whereas [ $^{14}\text{C}$ ]MeFH $_4$  fixation (via endogenous [ $^{14}\text{C}$ ]methionine synthesis) was used to indicate methyltransferase activity (17). The data in Table I show that, when measured in this way, the holo-

**TABLE I**  
Fixation of  $^{14}\text{C}$  from  $[^{14}\text{C}]\text{Propionate}$  and from  $[^{14}\text{C}]\text{MeFH}_4$  by Control and Mutant Fibroblasts

Cell line	$[^{14}\text{C}]\text{Propionate}$ fixation*	$[^{14}\text{C}]\text{MeFH}_4$ fixation*
Control ( $n = 4$ )‡	3.8–10.5	17–35
<i>cbl C</i> ( $n = 4$ )	0.2–0.7	0.1–1.5
<i>cbl D</i> ( $n = 2$ )	1.0–1.1	2.8–3.0

\* Nanoatoms  $^{14}\text{C}$  per milligram TCA-insoluble protein; figures given indicate the range of values obtained.

‡ Numbers in parentheses indicate the number of cell lines employed, each assayed several times.

enzyme activities of both Cbl-dependent enzymes in the two extant *cbl D* cell lines appear to be somewhat greater than those obtained from *cbl C* cell lines. Compared to the highest *cbl C* values obtained, mean *cbl D*  $[^{14}\text{C}]\text{propionate}$  fixation and  $[^{14}\text{C}]\text{MeFH}_4$  fixation levels were found to be 50% and 100% greater, respectively.

These results were corroborated by direct measurement of holoenzyme levels in cell free extracts (Table II). Whereas the fraction of total methyltransferase activity present as holoenzyme was less than 2.5% in *cbl C* cells, *cbl D* holomethyltransferase was found to be over 20%. Both of these values contrast with those from control-cell extracts, in which approximately 55% of total activity existed as holoenzyme. Total methyltransferase levels were also found to be generally lower in mutant cell extracts. These data are in close agreement with previous reports each of which examined only one or two cell lines (5, 6, 8, 19). Similarly, mutase holoenzyme activity appeared to be affected more severely in *cbl C*-cell extracts (not detectable, <0.2%) than in *cbl D* extracts (0.5–1.0%). Control cells were

**TABLE II**  
Activities of Cobalamin-Dependent Enzymes in Normal and Mutant Fibroblasts

	Cell line	Methyltransferase*	Mutase*
Holoenzyme activity	Control ( $n = 4$ )‡	49–68	18–67
	<i>cbl C</i> ( $n = 4$ )	<2	<4
	<i>cbl D</i> ( $n = 2$ )	10–16	8–15
Total enzyme activity	Control ( $n = 4$ )	110–119	898–2,483
	<i>cbl C</i> ( $n = 4$ )	49–83	815–2,407
	<i>cbl D</i> ( $n = 2$ )	51–66	1,438–2,512

\* Activities expressed as picomoles product (methionine or succinate) formed per minute per milligram protein; figures given indicate range of values obtained.

‡ Numbers in parentheses indicate the number of cell lines employed, each assayed several times.

found to contain between 3 and 8% holomutase under these growth conditions.

*Characteristics of methyltransferase and mutase from mutant fibroblasts.* The coordinate depression of both methyltransferase and mutase holoenzyme levels in *cbl C* and *cbl D* fibroblasts could result, theoretically, from an inability of the two apoenzymes to bind and retain Cbl as a result of a lesion affecting a shared subunit. Because such a defect might manifest itself as an alteration of the affinities of methyltransferase and mutase for their respective Cbl coenzymes, we measured these affinities in control and mutant cell extracts. As shown in Table III, neither mutase nor methyltransferase from *cbl C* or *cbl D* fibroblasts had  $K_m$ 's for their respective Cbl coenzymes which differed from those found in control cells. The affinity of methyltransferase for CN-Cbl was also determined because this Cbl species is almost as effective a cofactor as MeCbl (5) but must be methylated after binding to the enzyme (20). The  $K_m$ 's obtained for CN-Cbl were identical to those for MeCbl (Table III). However, it was found that CN-Cbl concentrations above 5  $\mu\text{M}$  were inhibitory, an observation of interest because most other investigators have reported using CN-Cbl at 50  $\mu\text{M}$  in vitro (5, 21–23). No such inhibition was observed at concentrations of MeCbl up to 400  $\mu\text{M}$ .

Since a mutation affecting a subunit common to both methyltransferase and mutase might not result in an alteration detectable in cell extracts as a  $K_m$  difference, the electrophoretic mobility of Cbl-dependent enzyme activity from mutant cells was also determined. Extracts

**TABLE III**  
Affinities of Cobalamin-Dependent Enzymes for Cobalamin Coenzymes in Normal and Mutant Fibroblasts

Cell line	Apparent $K_m$	
	Of methyltransferase for MeCbl or CN-Cbl	Of mutase for AdoCbl
	nM	
Control	400*	67*
<i>cbl C</i>	300*	45*
<i>cbl D</i>	400‡	40‡

The apparent  $K_m$ 's of methyltransferase and mutase for their Cbl coenzymes were determined in cell-free extracts by measuring enzyme activity as a function of Cbl concentration and analyzing the data with appropriate linear transformations. Cobalamin concentrations were determined spectrophotometrically at 367 nm after conversion of AdoCbl, CN-Cbl, and MeCbl to dicyanocobalamin (extinction coefficient = 30.4  $\text{mM}^{-1}$ ) by photolysis in 0.1 M potassium cyanide.

\* Values given are the means of results obtained from at least two cell lines.

‡ Values given were obtained with a single *cbl D* line.

were prepared from cells grown in medium that contained 1  $\mu\text{g/ml}$  OH-Cbl to increase endogenous holoenzyme content, because it was found that only holomethyltransferase activity could be recovered from gel slices. As shown in Fig. 4, no difference was observed in the electrophoretic profile of mutase and methyltransferase activity from *cbl C*'s relative to that from controls. Similarly, data discussed above (Fig. 3) indicated no detectable alteration in the mobilities of *cbl D* mutase and methyltransferase (as measured by [ $^{57}\text{Co}$ ]Cbl binding). Taken together, these results do not support the thesis that either of these mutations affects a possible subunit common to both Cbl-dependent enzymes.

## DISCUSSION

The intracellular binding of [ $^{57}\text{Co}$ ]Cbl to high molecular weight proteins has been observed to occur in a variety of cell types, including human fibroblasts (9), rat liver (10, 23), rabbit liver (11), Ehrlich ascites cells (24), Chinese hamster lung fibroblasts,<sup>3</sup> mouse L-cells,<sup>3</sup> and HeLa cells.<sup>3</sup> Recent data obtained with rodent liver labeled in vivo with [ $^{57}\text{Co}$ ]Cbl have suggested that the vast majority of this binding activity can be explained

by the attachment of [ $^{57}\text{Co}$ ]Cbl to the two Cbl-dependent enzymes, MeFH<sub>4</sub>:homocysteine methyltransferase and methylmalonyl-CoA mutase (10, 11). The data presented in this paper confirm and extend these findings to include cultured human fibroblasts. As in rat liver, we have shown that the proteins which bind intracellular [ $^{57}\text{Co}$ ]Cbl in fibroblasts are associated with mutase and(or) methyltransferase activities after both Sephadex G-150 chromatography and gel electrophoresis. Furthermore, although not shown, human fibroblast methyltransferase was also observed to co-elute with the single [ $^{57}\text{Co}$ ]Cbl-binding peak obtained when labeled cell extracts were chromatographed on DEAE-cellulose. Although subcellular fractionation of fibroblasts was not attempted (because of the lack of an efficacious method), it is most likely that, as in rat liver, the portion of [ $^{57}\text{Co}$ ]Cbl binding found on gels to be associated with methyltransferase activity is localized in the cytosol whereas that with mutase activity is in the mitochondrial fraction (10).

Aside from attesting to the generality of the results obtained with liver, the present data also have important consequences with respect to our understanding of the underlying defect in mutant cells deficient in the intracellular binding of [ $^{57}\text{Co}$ ]Cbl. Clearly, there is

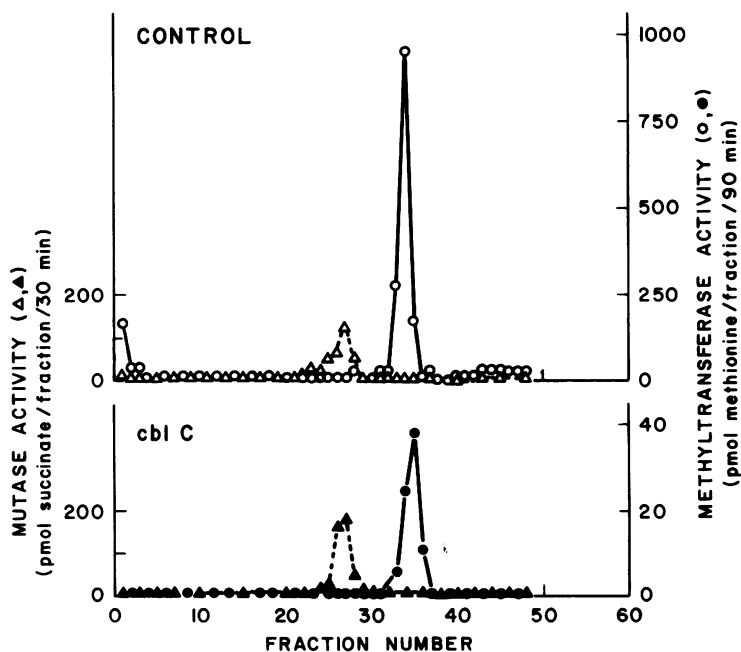


FIGURE 4 Comparison of the electrophoretic mobilities of methylmalonyl-CoA mutase activity ( $\blacktriangle$ ,  $\triangle$ ) and MeFH<sub>4</sub>:homocysteine methyltransferase activity ( $\bullet$ ,  $\circ$ ) from control and *cbl C*-fibroblast extracts. Gel slices were assayed in the presence of saturating amounts of Cbl (AdoCbl and MeCbl, respectively). However, omission of MeCbl from the reaction mixture was found not to decrease the methyltransferase activity detected, indicating that only holomethyltransferase activity could be recovered after electrophoresis. As indicated, both mutase and methyltransferase activities of mutant cells were electrophoretically indistinguishable from those of controls.

no longer the need to postulate the existence of a discrete Cbl-binding protein whose activity is impaired in *cbl C* and *cbl D* fibroblasts (9). Moreover, we have found no evidence to support the possibility that either of these mutations affects a putative subunit common to both methyltransferase and mutase, which might have explained their diminished ability to bind [<sup>57</sup>Co]Cbl. The affinities for Cbl coenzymes and electrophoretic mobilities of mutase and methyltransferase from mutant cells were unchanged relative to controls. The present results, of course, do not bear on the question of whether a common subunit exists, but only on the likelihood of its being affected by the *cbl C* or *cbl D* mutations. However, it is unlikely that these enzymes do share such a subunit, because experiments with human × rodent somatic cell hybrids have suggested that the methyltransferase enzyme is, in fact, a monomer.<sup>4</sup>

Why, then, is the intracellular binding of [<sup>57</sup>Co]Cbl deficient in *cbl C* and *cbl D* fibroblasts? Because controls were found to bind most of their [<sup>57</sup>Co]Cbl to methyltransferase (Fig. 2) and mutants were shown to have decreased levels of total methyltransferase activity in addition to diminished holoenzyme activity (Table II and [5, 6, and 19]), the possibility must be considered that a primary defect of this enzyme alone might result in such a phenotype. To explain the low levels of AdoCbl and mutase holoenzyme in these cells, however, binding to methyltransferase must then be presumed to be an obligatory intermediate step between the uptake of Cbl from the medium and its entry into the mitochondrion, the subcellular site of both AdoCbl synthesis and mutase activity (2, 10, 25–27). Although there is some kinetic evidence from rat liver which suggests that [<sup>57</sup>Co]Cbl is bound to methyltransferase before mutase (10), preliminary results using cultured fibroblasts appear to have indicated otherwise.<sup>3</sup> In addition, it has been possible to demonstrate directly the ability of *cbl C* methyltransferase to bind [<sup>57</sup>Co]Cbl in somatic cell hybrids formed from the fusion of *cbl C* and mouse fibroblasts.<sup>4</sup>

Because the endo-lysosomal system which mediates the specific uptake of TC II-bound Cbl has been shown to be normal in both *cbl C* and *cbl D* fibroblasts (28),<sup>3</sup> the most likely alternative possibility is that these mutations affect some enzymatic step(s) responsible for the metabolic conversion of [<sup>57</sup>Co]Cbl to a form which allows its binding to both apoenzymes. In that these cells are also deficient in the formation of Cbl coenzymes, it is probable that such a step occurs early in the intracellular metabolism of Cbl at a point common to the pathways of both MeCbl and AdoCbl biosynthesis. Little is known concerning these early events thus making it difficult to speculate further. However, it is

believed that Cbl, which enters the cell with its cobalt nucleus in an oxidation state of plus III (cob[III]-alamin), must undergo a stepwise reduction to cob(I)alamin before it is available as a substrate for coenzyme synthesis (29). Thus, these mutations might affect enzymes related to cob(III)alamin reductase activity.

Finally, the data presented in this paper demonstrate that *cbl D* fibroblasts can be distinguished biochemically from *cbl C* fibroblasts, as well as by genetic complementation analysis (3). While cells from both groups were found to be deficient in intracellular [<sup>57</sup>Co]Cbl binding and in the holoenzyme activities of both methyltransferase and mutase, *cbl D* fibroblasts were generally less severely affected relative to controls. The two brothers originally described by Goodman et al. (19) are thus far the only patients we have ascertained whose fibroblasts fall into the *cbl D* group. Whereas they are likewise less severely affected clinically compared to some of the patients expressing the *cbl C* mutation (5, 8, 30), the small number of cases involved precludes any general statement. None of the data obtained thus far, however, necessarily indicate that *cbl C* and *cbl D* fibroblasts express mutations affecting Cbl metabolism at distinct genetic loci, because in some cases complementation between mutant cell lines may occur as a result of interallelic (31), as opposed to intergenic (32) events. Indeed, both the clinical and cellular phenotypes associated with the *cbl D* mutation might also be associated with a “leaky” *cbl C* mutation. Resolution of this question must await the elucidation of the underlying biochemical defect in at least one of these two groups.

#### ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert assistance of Pamela Youngdahl-Turner and Marilyn Feldman in the preparation of this work.

This work was supported in part by research grants from the National Institutes of Health (AM 12579 and AM 09527).

#### REFERENCES

1. Gravel, R. A., M. J. Mahoney, F. H. Ruddle, and L. E. Rosenberg. 1975. Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 3181–3185.
2. Mahoney, M. J., A. C. Hart, V. D. Steen, and L. E. Rosenberg. 1975. Methylmalonicacidemia: Biochemical heterogeneity in defects of 5'-deoxyadenosylcobalamin synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 2799–2803.
3. Willard, H. F., I. S. Mellman, and L. E. Rosenberg. 1978. Genetic complementation among inherited deficiencies of methylmalonyl CoA mutase activity: Evidence for a new class of human cobalamin mutant. *Am. J. Hum. Genet.* **30**: 1–13.
4. Rosenberg, L. E. 1978. Disorders of propionate, methylmalonate, and cobalamin metabolism. In *The Metabolic*

<sup>4</sup> Mellman, I., P-f. Lin, F. H. Ruddle, and L. E. Rosenberg. Manuscript in preparation.



- Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 4th edition. 411-429.
5. Mudd, S. H., H. L. Levy, and G. Morrow. 1970. Deranged B<sub>12</sub> metabolism: Effects on sulfur amino acid metabolism. *Biochem. Med.* **4**: 193-214.
  6. Mahoney, M. J., L. E. Rosenberg, S. H. Mudd, and B. W. Uhlendorf. 1971. Defective metabolism of vitamin B<sub>12</sub> in fibroblasts from children with methylmalonicaciduria. *Biochem. Biophys. Res. Commun.* **44**: 375-381.
  7. Linnell, J. C., D. M. Matthews, S. H. Mudd, B. W. Uhlendorf, and I. J. Wise. 1976. Cobalamins in fibroblasts cultured from normal control subjects and patients with methylmalonic aciduria. *Pediatr. Res.* **10**: 179-183.
  8. Dillon, M. J., J. M. England, D. Gompertz, P. A. Goodey, D. B. Grant, H. A-A. Hussein, J. C. Linnell, D. M. Matthews, S. H. Mudd, G. H. Newns, J. W. T. Seakins, B. W. Uhlendorf, and I. J. Wise. 1974. Mental retardation, megaloblastic anaemia, methylmalonic aciduria and abnormal homocysteine metabolism due to an error in vitamin B<sub>12</sub> metabolism. *Clin. Sci. Mol. Med.* **47**: 43-61.
  9. Rosenberg, L. E., L. Patel, and A-C. Lilljeqvist. 1975. Absence of an intracellular cobalamin-binding protein in cultured fibroblasts from patients with defective synthesis of 5'-deoxyadenosylcobalamin and methylcobalamin. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 4617-4621.
  10. Mellman, I. S., P. Youngdahl-Turner, H. F. Willard, and L. E. Rosenberg. 1977. Intracellular binding of radioactive hydroxocobalamin to cobalamin-dependent apoenzymes in rat liver. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 916-920.
  11. Kolhouse, J. F., and R. H. Allen. 1977. Recognition of two intracellular cobalamin binding proteins and their identification as methylmalonyl CoA mutase and methionine synthetase. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 921-925.
  12. Shane, B., and E. L. R. Stokstad. 1975. Transport and metabolism of folates by bacteria. *J. Biol. Chem.* **250**: 2243-2253.
  13. Gupta, V. S., and F. M. Huennekens. 1967. Preparation and properties of crystalline 5-methyltetrahydrofolate and related compounds. *Arch. Biochem. Biophys.* **120**: 712-718.
  14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
  15. Willard, H. F., and L. E. Rosenberg. 1977. Inherited deficiencies of human methylmalonyl CoA mutase activity: Reduced affinity of mutant apoenzyme for adenosylcobalamin. *Biochem. Biophys. Res. Commun.* **78**: 927-934.
  16. Willard, H. F., L. M. Ambani, A. C. Hart, M. J. Mahoney, and L. E. Rosenberg. 1976. Rapid prenatal and postnatal detection of inborn errors of propionate, methylmalonate, and cobalamin metabolism: A sensitive assay using cultured cells. *Hum. Genet.* **34**: 277-283.
  17. Hoffman, R. M., and R. W. Erbe. 1976. High in vivo rates of methionine biosynthesis in transformed human and malignant rat cells auxotrophic for methionine. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 1523-1527.
  18. Davis, B. J. 1964. Disc electrophoresis—method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
  19. Goodman, S. I., P. G. Moe, K. B. Hammond, S. H. Mudd, and B. W. Uhlendorf. 1970. Homocystinuria with methylmalonic aciduria: Two cases in a sibship. *Biochem. Med.* **4**: 500-515.
  20. Burke, G. T., J. H. Mangum, and J. D. Brodie. 1970. Methylcobalamin as an intermediate in mammalian methionine biosynthesis. *Biochemistry.* **9**: 4297-4302.
  21. Kamely, D., J. W. Littlefield, and R. W. Erbe. 1973. Regulation of 5-methyltetrahydrofolate:homocysteine methyltransferase activity by methionine, vitamin B<sub>12</sub> and folate in cultured baby hamster kidney cells. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2585-2589.
  22. Ashe, H., B. R. Clark, F. Chu, D. N. Hardy, B. C. Halpern, R. M. Halpern, and R. A. Smith. 1974. N<sup>5</sup>-methyltetrahydrofolate:homocysteine methyltransferase activity in extracts from normal, malignant, and embryonic tissue culture cells. *Biochem. Biophys. Res. Commun.* **57**: 417-425.
  23. Pletsch, Q. A., and J. W. Coffey. 1972. Properties of the proteins that bind vitamin B<sub>12</sub> in subcellular fractions of rat liver. *Arch. Biochem. Biophys.* **151**: 157-167.
  24. Peirce, K., T. Abe, and B. Cooper. 1975. Incorporation and metabolic conversion of cyanocobalamin by Ehrlich ascites carcinoma cells in vitro and in vivo. *Biochim. Biophys. Acta.* **381**: 348-358.
  25. Fenton, W. A., L. M. Ambani, and L. E. Rosenberg. 1976. Uptake of hydroxocobalamin by rat liver mitochondria. *J. Biol. Chem.* **251**: 6616-6623.
  26. Fenton, W. A., and L. E. Rosenberg. 1978. Mitochondrial metabolism of hydroxocobalamin: Synthesis of adenosylcobalamin by intact rat liver mitochondria. *Arch. Biochem. Biophys.* **189**: 411-420.
  27. Frenkel, E. P., and R. L. Kitchens. 1975. Intracellular localization of hepatic propionyl CoA carboxylase and methylmalonyl CoA mutase in humans and normal and vitamin B<sub>12</sub>-deficient rats. *Br. J. Haematol.* **31**: 501-513.
  28. Youngdahl-Turner, P., L. E. Rosenberg, and R. H. Allen. 1978. Binding and uptake of transcobalamin II by human fibroblasts. *J. Clin. Invest.* **61**: 133-141.
  29. Fenton, W. A., and L. E. Rosenberg. 1978. Genetic and biochemical analysis of human cobalamin mutants in cell culture. *Annu. Rev. Genet.* **12**: 223-248.
  30. Mudd, S. H., B. W. Uhlendorf, K. R. Hinds, and H. L. Levy. 1970. Deranged B<sub>12</sub> metabolism: Studies of fibroblasts grown in tissue culture. *Biochem. Med.* **4**: 215-239.
  31. Chasin, L. A., and G. Urlaub. 1976. Mutant alleles for hypoxanthine phosphoribosyltransferase: Codominant expression, complementation, and segregation in hybrid Chinese hamster cells. *Somatic Cell Genet.* **2**: 453-467.
  32. Benzer, S. 1955. On the topography of the genetic fine structure. *Proc. Natl. Acad. Sci. U. S. A.* **41**: 344-354.