**Vitamin B\textsubscript{12}-dependent Replication of L1210 Mouse Leukemia Cells**

A MODEL SYSTEM FOR COBALAMIN-FOLATE INTER-RELATIONSHIPS*

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L1210 mouse leukemia cells were made cobalamin-deficient by propagation in a medium from which cyanocobalamin was omitted and fetal bovine serum (containing protein-bound cobalamins) was replaced by bovine serum albumin. These cobalamin-deficient cells exhibited a normal replication time of 12 h, provided that the medium contained excess folate or 5-formyltetrahydrofolate. The cells responded poorly, however, to 5-methyltetrahydrofolate unless exogenous cobalamin was added. A cobalamin dependency was also observed when low levels of folate or 5-formyltetrahydrofolate were used. With 5-methyltetrahydrofolate, optimal stimulation of growth was observed with free and transcobalamin-II-bound cobalamin at 4,000 pm and 2 pm, respectively. Under cobalamin-replete conditions, cells contained 2,000 to 4,000 molecules of cobalamin/cell, and in the deficient state, this value declined to approximately 180 molecules/cell. Cobalamin-deficient cells cultured in the absence of folate reached an arrested state from which limited replication could be induced by the addition of aquacobalamin; normal replication was induced by aquacobalamin plus 5-methyltetrahydrofolate. Results of this investigation are interpreted in terms of the requirement for tetrahydrofolate in cell replication and the production of this compound from folate and 5-formyltetrahydrofolate (via cobalamin-independent pathways) and from 5-methyltetrahydrofolate (via the cobalamin-dependent methionine synthetase).

Cobalamins are present in mammalian tissues at very low levels (1), but they play an important physiological role. Severe disorders (e.g. megaloblastic anemia, neuropathy, and suppressed growth) result from a deficiency of vitamin B\textsubscript{12} (2-4). Two cobalamin-dependent enzymes, methionine synthetase (EC 2.1.1.13) and methylmalonyl-CoA mutase (EC 5.4.99.2), are known to be present in a variety of mammalian cells (4), while a third enzyme, leucine 2,3-aminomutase, has been reported to occur in mammalian liver and human leukocytes (5).

The nature of the above clinical symptoms has suggested that cobalamin may be essential to the replication of mammalian cells by virtue of its participation in the reaction catalyzed by methionine synthetase (equation 1). Methionine, one product of the reaction, is an essential amino acid, while tetrahydrofolate, the other product, serves as the coenzyme for several key enzymatic reactions in cell replication (6). Attempts to demonstrate a cobalamin requirement for the replication of eukaryotic cells, however, have met with mixed success (7-15). In fact, vitamin B\textsubscript{12} was not included among the essential nutrients originally established by Eagle (16) for mammalian cell culture, although an absolute dependency on most other B vitamins is readily evident. Two factors contribute to this apparent anomaly: (a) Mammalian cells are generally propagated in media that include a high level of folate, which can supply tetrahydrofolate via a cobalamin-independent route (6); and (b) cultured cells require whole sera, which contain substantial amounts of protein-bound cobalamin (17).

The present investigation provides direct evidence for the involvement of cobalamin in the replication of L1210 mouse leukemia cells. In order to demonstrate this dependency, however, the cells had to be adapted to grow on serum albumin instead of whole serum in order to bring the intracellular cobalamin content down to a very low level. Cell propagation under these conditions was insured by providing a high level of folate, but when the latter was replaced by 5-methyltetrahydrofolate, there was an absolute requirement for exogenous cobalamin for continued growth. L1210 cells cultured in this manner thus provide a convenient model system for the examination of cobalamin-folate interrelationships.

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**EXPERIMENTAL PROCEDURES**

**Materials**—The following were obtained from the indicated commercial sources: crystalline bovine serum albumin and folate, Sigma; 5-formyltetrahydrofolate, ICN-K & K; [1\textsuperscript{4}C]cyanocobalamin, Ame- sham; penicillin, streptomycin, complete RPMI 1640 medium, and "deficient," double strength RPMI 1640 medium lacking folate, cyanocobalamin, and methionine), Associated Biomedic Systems; feta bovine serum, horse serum, and human serum, Grand Island Biological; CM-cellulose, Whatman; TEAE-cellulose, Accurate/ Serva; Sephadex G-150, Pharmacia.

Aquacobalamin was obtained by photolysis of methylcobalamin (18) and purified by phenol extraction and chromatography on CM-cellulose (19). Methylcobalamin was prepared by treatment of cyanocobalamin with potassium borohydride and methyl iodide (20) and purified as described above. 5-Methyltetrahydrofolate was synthesized by the method of Gupta and Huennekens (21), except that an additional step was used, namely, chromatography on TEAE-cellulose with elution via a gradient of ammonium acetate (0.1 M → 0.4 M) that also contained mercaptoethanol (20 mM). Bovine serum albumin was dissolved in water (70 mg/ml) and dialyzed against water for 4 h at 4°C.
Cobalamin Requirement for L1210 Cell Replication

Standard Culture Conditions—Stock L1210 cells were maintained in complete RPMI 1640 medium (Medium A) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (50 μg/ml). Inocula were added to 10 ml of medium (to achieve a density of 2 to 6 × 10⁶ cells/ml) in Corning polystyrene flasks, and the latter were incubated at 37°C in a humidified atmosphere (5% CO₂, 95% air). When the determination of Coulter count parameters showed that transfers were made every 2 or 3 days. Cultures were examined periodically for contamination by mycoplasma using the uridine phosphorylase assay (22).

Cobalamin-deficient Cells (Method I)—Serum-grown L1210 cells (see above), washed by centrifugation (200 g; 5 min) with folate- and cobalamin-deficient RPMI 1640 (Medium B), were transferred to Medium B supplemented with folate (100 μM) and bovine serum albumin (7 mg/ml). Other culture conditions were the same as those described above. After 2 to 3 days, the cells began to replicate slowly, and by 14 days they had regained their initial generation time of 12 h. Further maintenance on this regimen for an additional 14 days resulted in cells that contained virtually no cobalamin (<10 molecules/cell); the generation time, however, remained constant at 12 h.

Cobalamin-deficient Cells (Method II)—This procedure was similar to Method I, except that folate was replaced with 5-methyltetrahydrofolate (10 μM). In this instance, the cobalamin deficiency resulted in a gradual lengthening of the generation time to approximately 35 h. For both methods the time required to develop deficient cells depends upon the extent to which the serum albumin and medium are contaminated with cobalamins.

Growth Requirements for Folate and Cobalamin Compounds—Cells were grown by centrifugation (as described above) in order to avoid carrying over components from the original medium and then added to Medium B, supplemented with folate and cobalamin compounds as indicated. Cell growth was measured during the logarithmic phase (density <10⁵ cells/ml). In these experiments, concentrations of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate refer to the racemic mixture.

Purification of Transcobalamin-II from Human Serum—Transcobalamin-II was purified from 2,500 ml of pooled human sera essentially according to the method of Puutula and Graabeck (23); exogenous cobalamin was not added. The procedure involved repeated chromatography on CM-cellulose, followed by passage through Sephadex G-150. Apo-transcobalamin-II was located by testing eluted fractions for their ability to bind [⁵⁷Co]cyanocobalamin (23). The final purification (17.2 mg of protein), purified approximately 6,000-fold, contained 29.7 μg of apo-transcobalamin-II (determined by cobalamin-binding capacity) and 16.3 μg of holo-transcobalamin-II (determined by assay of bound cobalamin). When an aliquot of this preparation labeled with [⁵⁷Co]cyanocobalamin was passed through a calibrated Sephadex G-150 column, a single radioactive peak was observed (molecular weight 39,000). For growth experiments, transcobalamin-II was sterilized by passage through a cellulose acetate membrane (0.2 μm), and no additional cobalamin was added to saturate the apo-form.

Determination of Cobalamin—Washed cells (1 to 20 × 10⁶, depending upon the cobalamin content) were treated for 15 min with 2 ml of 0.3 mM KCN, 44 mM glutamic acid, pH 3.3, in a boiling water bath. After centrifugation (20 min; 2,000 g), the supernatant was saved, and the extraction was repeated twice with 1 ml portions of the above solution. The cobalamin content of each supernatant was determined by a radioisotope dilution method (24) using a commercially available kit (Pharmacia). For cobalamin-deficient cells, it was necessary to extend the standard curve to a 10-fold lower concentration (2.5 ng/ml). Efficiency of extraction, usually >95%, was reduced to approximately 80% for cobalamin-deficient cells. Recovery of cyanocobalamin added in 10-fold excess prior to extraction was generally 90%, but this value dropped to 60% for the very low levels seen in cobalamin-deficient cells.

Other Methods—Protein concentration was measured by the method of Lowry et al. (25) with bovine serum albumin as the standard. Radioactivity of [⁵⁷Co] was determined with a γ counter (Nuclear Chicago, model 1185B).

RESULTS

Methionine Requirement of L1210 Cells—In order to restrict the role of the cobalamin-dependent methionine synthase to generation of methyltetrahydrofolate, it was necessary to provide an excess to the other reaction product, methionine (see equation 1). The quantitative requirement for methionine was determined empirically by propagating cells in a mixture of dialyzed fetal bovine serum, methionine-deficient RPMI 1640 medium, and increasing amounts of methionine. Optimal growth was reached at 40 to 60 μM methionine. The same result was also obtained (Fig. 1) when the following modifications were made in the experimental conditions: (a) bovine serum albumin was substituted for fetal bovine serum; (b) folate was replaced by 5-methyltetrahydrofolate; and (c) cobalamin-deficient cells were used. In all subsequent work, methionine was included in the culture medium at 100 μM (i.e. its concentration in RPMI 1640).

Adaptation of L1210 Cells for Growth on Serum Albumin—The L1210 cells used to initiate this investigation were obtained from a stock culture that had been propagated continuously on 5% fetal bovine serum plus complete RPMI 1640 medium (Medium A); the latter contains 3.7 nm cyanocobalamin. For the projected experiments, it was necessary to develop cobalamin-deficient cells. This was accomplished by deleting cyanocobalamin from the culture medium and by avoiding the use of whole serum, which contains protein-bound cobalamin. When serum was deleted, however, the inoculum cells failed to multiply and began to disintegrate. Addition of bovine serum albumin prevented cell degeneration and, after 2 to 3 days of quiescence, replication gradually resumed. When inoculated at higher initial densities (up to 5 × 10⁶ cells/ml), the lag period was eliminated. After 2 weeks, the albumin-adapted cells achieved the previous generation time of 12 h. These cells tended to aggregate and to adhere to the vessel, but after several months of serial transfer, these undesirable characteristics disappeared; morphology and pathogenicity (in BDF, mice) were then indistinguishable from cells sustained in whole serum.

Growth of the adapted cells as a function of the concentration of serum albumin is shown in Fig. 2. The optimal effect was reached at approximately 7 mg/ml, and all subsequent experiments were performed with serum albumin present at this concentration.

Detection of a Cobalamin Requirement for Cell Replication—Cobalamin-replete cells, growing routinely in Medium A plus fetal bovine serum were found, via radioisotope dilution assay, to contain 7.2 pg of cobalamin/10⁶ cells; this corresponds to an average value of 3,200 molecules of cobalamin/
cell. Cells grown under conditions of minimal exposure to cobalamin (see above) eventually reached a stage in which the endogenous level was barely detectable (<10 molecules/cell). Although these cells were maintained by growth on folate (Method I, "Experimental Procedures"), cells equally deficient in cobalamin could also be obtained by prolonged growth on 5-methyltetrahydrofolate (Method II). In the latter instance, however, the replication time gradually increased to 35 h as the cells became cobalamin-deficient. In all subsequent experiments, "cobalamin-deficient" cells (prepared by Method I, "Experimental Procedures"), cells equally deficient in cobalamin when cells were grown on excess folate or 5-formyltetrahydrofolate. When the concentrations of these latter compounds were lowered, however, a cobalamin dependency was clearly evident (Fig. 4, A and B). These data confirm and extend the previous results by showing that added cobalamin is unnecessary when the concentrations of folate and 5-formyltetrahydrofolate are greater than 10 and 0.1 μM, respectively. In the presence of the cobalamin, optimal growth was reached at lower concentrations of these folate substrates (approximately 1 and 0.01 μM). Similar concentration dependencies have been reported previously for serum-grown L1210 and L5178Y cells (13, 26–28).

It was of interest to ascertain whether the cobalamin requirement for cells grown on 5-methyltetrahydrofolate (Fig. 3B) could also be diminished by increasing the concentration of this folate compound. However, even at 100 μM 5-methyltetrahydrofolate, growth was negligible in the absence of the cobalamin (Fig. 5). In the presence of cobalamin, growth was considerably less than in its presence (panel B). In the latter experiment, the same degree of stimulation seen with free aquacobalamin could be achieved by the addition of 5% fetal bovine, horse, or human serum (data not shown).

Further Examination of the Cobalamin Requirement for Growth of Cells upon Various Folate Compounds—In the preceding experiment (Fig. 3A), there was no requirement for cobalamin when cells were grown on excess folate or 5-formyltetrahydrofolate. In the presence of this folate compound, growth was negligible in the absence of the cobalamin (Fig. 5). In the presence of cobalamin, growth was

![Fig. 2. Growth of cells on bovine serum albumin. L1210 cells (initial density, 5 × 10⁴ cells/ml) were grown in Medium B, supplemented with 100 μM folate and the indicated concentrations of dialyzed bovine serum albumin. Results are expressed as growth relative to that in the system containing albumin at 10 mg/ml. Time, 53 h.](image)

![Fig. 3. Effect of aquacobalamin upon growth of cobalamin-deficient cells in the presence of folate, 5-formyltetrahydrofolate, or 5-methyltetrahydrofolate. A, cobalamin-deficient cells (Method I) were grown in Medium B supplemented with serum albumin and 100 μM folate, and in the presence (●) or absence (○) of 10 nM aquacobalamin. Cell densities were determined at the indicated times. Identical data were obtained when folate was replaced by 1 μM 5-formyltetrahydrofolate. B, experimental conditions as in A, except that folate was replaced by 10 μM 5-methyltetrahydrofolate.](image)

![Fig. 4. Concentration dependence upon folate or 5-formyltetrahydrofolate for cells growing in the presence and absence of aquacobalamin. Experimental conditions as in Fig. 3, except that the concentrations of folate (panel A) and 5-formyltetrahydrofolate (panel B) were varied as indicated. Time, 72 h.](image)

![Fig. 5. Concentration dependence upon 5-methyltetrahydrofolate for cells growing in the presence and absence of aquacobalamin. Cobalamin-deficient cells (obtained by Method II) were grown in Medium B supplemented with serum albumin and the indicated concentration of 5-methyltetrahydrofolate in the presence (●) or absence (○) of aquacobalamin (10 nM). Initial density, 6 × 10⁴ cells/ml. Time, 72 h.](image)
maximal at concentrations greater than approximately 0.2 μM; a similar concentration dependence has been reported previously for L5178Y cells (13).

Quantitation of the Cobalamin Requirement for Cell Replication—Information derived from the preceding experiments was used to design a system for determining the quantitative requirement for exogenous cobalamin in the replication of L1210 cells. Cells with a very low cobalamin content were used to inoculate a medium containing 5-methyltetrahydrofolate and variable amounts of aquacobalamin (Fig. 6A). These cells grew very poorly in the absence of added cobalamin, but in its presence, growth was nearly linear with low concentrations and reached a plateau at about 4 nm. Similar results were obtained when aquacobalamin was replaced by cyanocobalamin, methylcobalamin, or adenosylcobalamin (data not shown).

In the above experiment, exogenous cobalamin was provided in the free form. The enormous advantage of transcobalamin-II in promoting cellular uptake of cobalamin and, in turn, stimulating cell replication is shown in Fig. 6B. In this instance, the maximal effect was achieved at a cobalamin concentration of only 2 μM. This was about 1/2000 the level of free aquacobalamin required (cf. Fig. 6A).

Restoration of Cobalamin Levels in Depleted Cells—Cells grown on either folate or 5-methyltetrahydrofolate in medium B and serum albumin lost essentially all of their endogenous cobalamin (Table I, Experiment 1). Values for the lowest levels must be regarded as approximate, since their measurement necessitated the use of a large number of cells and small volumes of extractant to obtain a marginal response in the assay. Addition of increasing amounts of aquacobalamin to the medium led to the progressive restoration of the original level of endogenous cobalamin (Experiment 2). The same result could also be achieved by transferring the cobalamin-deficient cells to fetal bovine serum, and an even higher level was reached by inclusion of aquacobalamin with the whole serum (Experiment 3).

The above data were used to obtain an approximate value for the minimal amount of intracellular cobalamin necessary for optimal replication in the presence of 5-methyltetrahydrofolate. It was shown previously (Fig. 6A) that the latter effect could be achieved when the external concentration of cobalamin was about 2 nm, which would produce an intracellular concentration of 180 molecules/cell (Table I).

Cobalamin-dependent Resumption of Growth in Folate-starved Cells—Cobalamin-deficient cells propagated on folate were transferred to a medium containing a suboptimal concentration of this compound. After 72 h, these cells were used as inocula for the experiment shown in Fig. 7. In a folate- and cobalamin-free medium (Medium B), replication ceased after 80 h; only 1-3 doublings occurred during that period (curve 1). The presence of aquacobalamin alone caused a more rapid and prolonged response (curve 2), while optimal growth was achieved by the addition of 5-methyltetrahydrofolate along with aquacobalamin (curve 3). Extending these results, growth-arrested cells (cf. curve 1) could be induced to undergo limited replication by the addition of aquacobalamin alone (curve 4) or to achieve optimal growth by the addition of aquacobalamin plus 5-methyltetrahydrofolate (curve 5). Similar results (data not shown) were obtained when the cells were maintained initially on 5-formyltetrahydrofolate, rather than folate.

**DISCUSSION**

Vitamin B₁₂ has been reported previously to stimulate replication of some mammalian cells *in vitro* (7, 10, 12–15), but
the results have varied considerably with respect to the degree of stimulation and the cobalamin concentrations required to achieve these effects. In other instances, attempts to detect a cobalamin requirement were unsuccessful (8, 9, 11, 16). In the present investigation, a key role for cobalamin in the replication of L1210 mouse leukemia was established, and a detailed examination has been made of factors that govern the cobalamin dependency. It was necessary to employ carefully controlled experimental conditions in order to obtain these results. Two factors were crucial: (a) cobalamin-deficient cells had to be developed; and (b) deficient cells had to be tested for cobalamin-dependent replication in a well defined medium.

The first objective was achieved by deleting cyanocobalamin from RPMI 1640 medium and by replacing whole serum (which contains endogenous cobalamin bound to cobalamin-binding proteins, including transcobalamin-II) by serum albumin. Although the samples of serum albumin used in this investigation were not entirely devoid of cobalamin (5 to 7 pg/mg of protein), this contaminant appeared to be bound to proteins incapable of effecting its transport into cells. Cells were propagated on serum albumin until the initial intracellular cobalamin (3,200 molecules/cell) had become highly attenuated (<10 molecules/cell) (Table 1). During the several weeks required to reach this deficient state, continuous proliferation of the cells (with a normal doubling time of 12h) was insured by providing a high level of folate, whose conversion to the coenzyme, tetrahydrofolate, proceeds via a cobalamin-independent pathway (Fig. 8). 5-Methyltetrahydrofolate could replace folate, but the dependence of its metabolism upon a cobalamin enzyme resulted in a gradual increase in doubling time to approximately 35 h as the cells became deficient. The second objective was accomplished by examining the growth of deficient cells in the presence of 5-methyltetrahydrofolate supplemented with increasing amounts of aquacobalamin. Maximum stimulation of growth was seen with free aquacobalamin at 4 nM, but this value decreased dramatically to 2 pm when the cobalamin was presented as the transcobalamin-I I complex (Fig. 6, A and B). A requirement for cobalamin could also be demonstrated when limiting concentrations of folate or 5-formyltetrahydrofolate replaced 5-methyltetrahydrofolate.

Several aspects of the experimental design require additional comment. (a) Although whole serum has been used traditionally for the propagation of mammalian cells, several types of cells can be grown on serum albumin (29, 30). One possible basis for the effectiveness of this substitution is that highly purified serum albumin, even after extensive dialysis, retains sufficient amounts of various factors necessary for cell proliferation. Fortunately for the present purpose, functional transcobalamin-II does not appear to be one of these contaminants. (b) Most media used for propagation of mammalian cells contain folate, e.g. 2.3 pm in RPMI 1640), even though 5-methyltetrahydrofolate is the principal circulating form of this vitamin in mammals (31). Since utilization of folate does not involve any cobalamin-dependent step, in vitro studies performed in the presence of this compound are unlikely to detect a cobalamin requirement for replication. (c) In most of the experiments in this investigation, cobalamin was provided to the cells in the free state rather than in a complex with transcobalamin-II. The latter material, which greatly enhances the uptake of cobalamins (32, 33), is difficult to obtain in highly purified form and in sufficient quantity to be used routinely in cell culture systems. Although required at a relatively high concentration (cf. Fig. 6A), free aquacobalamin appears to be quite adequate. This is consistent with the observation of Hall et al. (34) that [15]cyanocobalamin is taken up slowly by HeLa cells in the absence of transcobalamin-II. The mechanism by which free cobalamin enters cells is not yet known, but it may involve passive diffusion, endocytosis of a complex containing cobalamin bound to a protein other than transcobalamin-I, or internalization of transcobalamin-II. (d) L1210 cells were provided with 100 pm methionine (an excess over that required for optimal replication) (Fig. 1) to insure that its production via methionine synthetase (equation 1) would not be a limiting factor. This procedure also obviated the need for homocysteine in the medium, since this substrate for the synthetase must have been generated from methionine via adenosylmethionine and adenosylhomocysteine. It has generally been found that normal cells, but not malignant cells, continue to proliferate when methionine is replaced by homocysteine (35, 36). Consistent with this observation, the L1210 mouse leukemia cells used in this investigation could not be propagated on homocysteine (even when supplemented with cobalamin and 5-methyltetrahydrofolate), although they contain measurable levels of cobalamin-dependent methionine synthetase (37). Thus, unlike the situation in certain other cells (10, 14), methionine production in L1210 cells is not a suitable parameter for evaluating a cobalamin requirement.

The present results are readily understood in terms of the participation of the folate coenzyme, tetrahydrofolate, in cell replication. One-carbon adducts of this coenzyme are involved in the synthesis of purine nucleotides and the pyrimidine nucleotide thymidylate and, hence, play a key role in DNA synthesis (6). An adequate amount of newly acquired tetrahydrofolate is needed, therefore, each time a cell undergoes division. Three different routes for generating tetrahydrofolate, shown in Fig. 8, are relevant to the present investigation: (a) from folate via NADPH-dependent dihydrofolate reductase; (b) from 5-formyltetrahydrofolate via transformation to glutamate or ATP-dependent conversion to 5,10-methylene tetrahydrofolate and subsequent loss of the C1 group; and (c) from 5-methyltetrahydrofolate via cobalamin-dependent methionine synthetase (equation 1). It is not surprising, therefore, that cobalamin-depleted cells grow poorly on 5-methyltetrahydrofolate (Figs. 3B and 5) but show unimpaired growth on adequate levels of folate or 5-formyltetrahydrofolate (Fig. 4, A and B). The failure of cobalamin-depleted cells to replicate well on low levels of the latter two compounds (Fig. 4, A and B) suggested that both were being diverted via 5,10- methylenetetrahydrofolate to an inert pool of 5-methyltetrahydrofolate (Fig. 8); data supporting this conclusion will be

![Fig. 8. Cobalamin-dependent and independent pathways for the interconversion of folate coenzymes. Abbreviations: F., folate; FH2, dihydrofolate; FH4, tetrahydrofolate; CHFH4, 5-methyltetrahydrofolate; HCOPH4, 5-formyltetrahydrofolate; CH2FH4, 5,10-methylenetetrahydrofolate. Other abbreviations are standard.](image-url)
Covalamin Requirement for L1210 Cell Replication

published separately. This emphasizes the importance of methionine synthetase in the replication of mammalian cells, particularly since these cells in vitro are nurtured primarily by 5-methyltetrahydrofolate.

Optimal growth of cells required quite different concentrations of folate and 5-formyltetrahydrofolate (e.g., 1.0 and 0.01 μM in covalamin-replete cells (Fig. 4, A and B)), which can be explained in terms of cellular uptake of these compounds. Kc values (concentrations necessary for half-maximal rates of uptake) favor 5-formyltetrahydrofolate by about 50-fold and, since both have the same Vmax value (38), the relative rates of uptake at low concentrations would be the ratio of their Kc values. After internalization, conversion to tetrahydrofolate is much faster than uptake (39). These considerations are probably also applicable to 5-methyltetrahydrofolate, but it is difficult to determine the actual concentration of this compound required to support optimal growth owing to its lability in cultures.

Studies by Herbert and Zalusky (40) and by Noronha and Silverman (41) have shown that 5-methyltetrahydrofolate accumulates in the serum of covalamin-deficient patients. These investigators proposed the "methyl trap" hypothesis, which stated that the metabolism of this reduced folate compound could occur only via the covalamin-dependent methionine synthetase (equation 1). The present studies provide further substantiation for this concept. Since covalamin-deficient animals are difficult to obtain (2), the in vitro L1210 model provides a convenient and relevant system for examining the inter-relationship of covalamins and folates. It will be of interest to see whether the present techniques will allow a covalamin dependency for replication to be demonstrated in other eukaryotic cells.

Results obtained with the L1210 system also correlate well with clinical findings. (a) Folate concentrations optimal for covalamin-replete cells are suboptimal in covalamin-deficient cells (Fig. 4A). This is consistent with the clinical observation that covalamin therapy can relieve folate deficiency, and vice versa (42, 43). (b) Covalamin-deficient cells cannot utilize 5-methyltetrahydrofolate unless covalamins are also provided (Fig. 5). In covalamin-deficient patients, 5-methyltetrahydrofolate must be used in conjunction with covalamin to restore biochemical parameters (e.g., deoxyuridine suppression test (44)) to normal values. (c) Covalamin bound to transcobalamin-II can be replaced by a 2,000-fold higher concentration of free covalamin (Fig. 6, A and B). Similar findings have been reported for patients with congenital deficiency of transcobalamin-II, who responded to pharmacological doses of the vitamin (34).

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