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Metabolism of Cyanocobalamin by L-1210 Leukemic Lymphoblasts (38813)

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The cellular uptake of vitamin B-12 is a calcium-dependent process mediated by two of the recognized protein binders of vitamin B-12, gastric intrinsic factor (IF) and plasma transcobalamin II (TCII). Although the mechanism of transmembrane transport remains obscure there is evidence in a number of systems that the entire protein B-12 complex may enter the cell.

Pletch and Coffey (1) studied the uptake of Vitamin B-12 by rat liver *in vivo* after intracardiac injection of radiolabelled vitamin. They noted that intracellular B-12 was associated with a protein having the same molecular weight as TCII and suggested pinocytosis of the TCII–B-12 complex. Newmark (2) studied B-12 localization in fractionated rat kidney and also proposed entrance of TCII-bound B-12 by pinocytosis. Toporek *et al.* (3) observed that hog intrinsic factor facilitated B-12 absorption by isolated perfused rat liver with subsequent transfer of the vitamin to bile. The B-12-containing bile facilitated absorption of vitamin B-12 in a second perfused liver. This observation suggested cellular uptake of a protein B-12 complex in view of the fact that bile per se does not enhance liver uptake of vitamin B-12.

We have previously investigated the uptake of Vitamin B-12 from plasma employing an *in vitro* system of isolated murine L-1210 leukemic lymphoblasts (4). The majority of the B-12 taken up by the cells was found in the cytoplasmic soluble phase bound to a protein having the physical properties of TCII and the capacity to deliver B-12 to fresh L-1210 cells. This observation was consistent with the possibility that the entire TCII B-12 complex enters the cell. We have furthered these observations in the present study by investigating the fate of vitamin B-12 after uptake by L-1210 cells.

Materials and Methods. Cell preparation. Propagation and isolation of L-1210 lymphoblasts has previously been described (4). Cells were extracted in normal saline 6 days after intraperitoneal injection of 10^5 L-1210 cells into female BDF_1 mice. The intraperitoneal exudate was centrifuged (1650*g* for 10 min) and any contaminating erythrocytes removed by brief exposure to hypotonic saline. The cells were then washed twice with Fischer's medium for leukemic cells of mice (G1BCO 147:G, Grand Island Biological Company, Grand Island, NY) made $10^{-2}M$ in $CaCl_2$ (Fischer's–$CaCl_2$). A 4% (packed cell volume) suspension in Fischer's $CaCl_2$ was kept at 4°C (5–15 min) prior to use. Stained smears of the cells demonstrated 99%–100% L-1210 lymphoblasts which excluded trypan blue for up to 6 hr at 37°C.

Plasma preparation. Human EDTA plasma (Vacutainer, BD4739) was obtained from a healthy donor. The unsaturated B-12 binding capacity (UBBC) was determined by adding an excess (3 ng/ml) of ^{57}Co B-12 (Amersham/Searle Corp., sp act 50–150 mCi/mg), followed by dialysis against normal saline or gel filtration on Sephadex G-25 (UBBC 1.5 ng/ml).

Endogenous L-1210 B-12 binding protein. Approximately 10^9 L-1210 cells in Fischer's $CaCl_2$ were disrupted with 50 double strokes in a Dounce glass homogenizer and clarified by centrifugation. The clear cellular homogenate (20 ml) was concentrated to 2 ml by ultrafiltration employing an Amicon UM-10 membrane. To this concentrated sample was then added 1 ng of ^{57}Co B-12. After 30-min incubation at 37°C, an aliquot (0.5 ml) was subjected to gel filtration on Sephadex G-25. The remaining sample was incubated in duplicate with 10^7 L-1210 cells at 37°C for 2 hr as previously described (4). Normal saline and recalcified human plasma served as controls.

Cell uptake. Approximately 10^9 L-1210

cells in Fischer's $CaCl_2$ were incubated with 10 ml recalcified human plasma subsaturated with 10 ng ^{57}Co B-12 for 2 or 6 hr at 37°C. The cells were then separated and washed three times with $10^{-2}M$ EDTA–normal saline, suspended in distilled water, disrupted with 50 double strokes in a Dounce glass homogenizer, and clarified by centrifugation. The incubation supernatants and clear cellular homogenates were then dialyzed overnight against distilled water. Plasma subsaturated with ^{57}Co B-12 (1 ng/ml) was incubated in the absence of cells to serve as a control.

Extraction and separation of cobalamins. Cobalamins were extracted from the dialyzed incubation supernates, cellular homogenates, and controls by the hot ethanol procedure described by Andstrand and Stahlberg (5) as modified by Linnell *et al.* (6). In brief, each sample (20 ml) was mixed with absolute ethanol (80 ml) and heated to 80°C for 20 min. The mixture was then cooled in an ice bath and filtered. Alcohol was removed from the filtrate in a rotary evaporator gradually increasing the temperature from 25°C to 40°C. The aqueous residue was then washed three times with ether (20-ml aliquots) and residual ether was removed by evaporation. The aqueous residue was then added to phenol containing 15% water (80 ml) and shaken vigorously. The aqueous phase was removed and the phenol layer washed three times with water (20-ml aliquots). The phenol layer was then mixed with acetone (70 ml) and ether (210 ml) and shaken with water (10 ml) for extraction of the cobalamins. The aqueous phase was then washed three times with equal volumes of ether. Residual ether was removed by evaporation. The samples were further concentrated by evaporation to a final volume of 1 ml. The extracted cobalamins were then separated by chromatography on SP-Sephadex C-25 as described by Tortolani *et al.* (7). The ion exchanger was initially equilibrated with sodium acetate buffer (0.05 *M*, pH 5.0) followed by repeated washing on the column with distilled water. All samples were cochromatographed with a mixture containing 100 μg each of cyanocobalamin, adenosylcobalamin, methylcobalamin (all Calbiochem), and hydroxycobalamin (Sigma) standards.

The optical density of each fraction was scanned between 365 and 335 nm, a range chosen to encompass the λ max of each standard. The radioactivity of each fraction was determined in an automatic well scintillation counter (Nuclear-Chicago Model 1185) calibrated with ^{57}Co B-12 samples of known activity. All incubation, extraction, and separation procedures were carried out in the dark or with a red safelight to prevent photolytic degradation of B-12 analogs.

Results and Discussion. We have previously demonstrated that L1210 leukemic lymphoblasts take up vitamin B-12 when it is bound to plasma TCII (4). After homogenization of the L1210 cells the majority of the vitamin B-12 was in the cytoplasmic soluble phase apparently still bound to TCII although the possibility that the cells contained an endogenous TCII-like protein was not excluded. Preliminary experiments in our laboratory (unpublished data) indicate that isolated mitochondria demonstrate enhanced uptake of vitamin B-12 bound to TCII. A portion of such TCII bound vitamin B-12 is converted to coenzyme form by isolated mitochondria. In the present study we have investigated the fate of protein-bound vitamin B-12 after incubation with intact L-1210 cells.

Endogenous L-1210 B-12 binding protein. In an attempt to demonstrate the possible presence of endogenous B-12 binders with the characteristics of TCII in L1210 cells, cellular homogenates were incubated with vitamin B-12 and subjected to gel filtration on Sephadex G-25 as well as incubation with fresh L1210 cells. These experiments demonstrate that none of the radioactive B-12 was protein bound after such incubation (Fig. 1). Furthermore, the cellular homogenate contained no substances able to deliver radioactive B-12 to fresh L1210 cells (Table I). Thus, L1210 cells do not appear to contain vitamin B-12 binding proteins in the cytoplasmic soluble phase, nor does the homogenate enhance vitamin B-12 uptake in fresh cells.

Cellular conversion of cyanocobalamin.

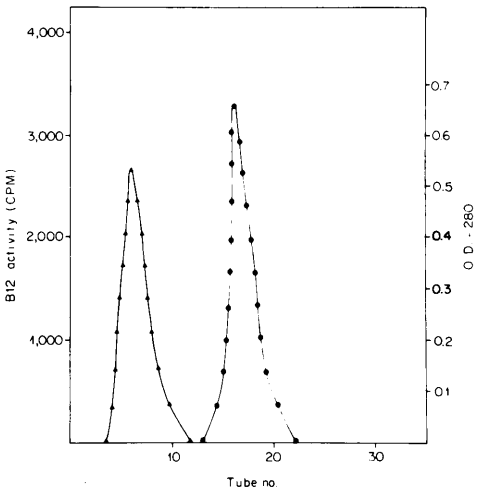

FIG. 1. Elution from Sephadex G-25 of a homogenate of L-1210 cells preincubated with 1 ng ^{57}Co-B_{12} (column size 0.9 × 25 cm, sample size 0.5 ml, fraction size 1 ml, eluant 0.5 *M* NaCl, flow rate 6 ml/hr). The clear separation of the OD 280 (▲) from the radioactivity (●) demonstrates lack of protein binding.

TABLE I. ^{57}Co B-12 UPTAKE BY L-1210 CELLS

Media[a]	Uptake (pg $B_{12}/10^7$ cells)
L-1210 homogenate	1.1
Saline	1.0
Human plasma	18.0

[a] All incubation media contain 10^{-2} *M* $CaCl_2$.

L-1210 cells were then incubated for 2 and 6 hr with ^{57}Co B-12 bound to human plasma. Dialysis of the incubation supernate and cellular homogenate resulted in negligible loss of radioactivity again suggesting that the vitamin B-12 remains protein bound. After hot ethanol extraction the various cobalamins were separated on SP-Sephadex C-25. A typical elution profile is shown in Fig. 2. The percentages of each of the cobalamins present in the 2- and 6-hr incubation supernates, cellular homogenates, and the plasma control are presented in Table II. Plasma bound B-12 incubated in the absence of cells was found to remain entirely in the cyano form. After 2-hr incubation with L1210 cells, however, approximately 6% of the B-12 was converted to other forms. After 6 hr approximately 10% was converted. Adenosylcobalamin is the major conversion product at 2 hr with methylcobalamin appearing in increased quantities at 6 hr. Peters and Hoffbrand (8) demonstrated conversion of cyanocobalamin to adenosylcobalamin in the guinea pig ileum and presented evidence that this conversion occurred in mitochondria. In their experiments approximately 20% of the vitamin was converted to a coenzyme form but a large proportion of the cyanocobalamin was absorbed unchanged. After 48 hr of incubation with L1210 cells in tissue culture, DiGirolamo *et al.* (9) found that added cyanocobalamin had been converted to adenosyl B-12 (36%), hydroxy-B-12 (28%), and methyl-B-12 (6%), while 30% remained in the cyano form. Our results after short-term incubation *in vitro* show similar trends, although no hydroxycobalamin was detected in our samples. This B-12 form may occur only after prolonged incubation, or may have resulted from hydrolysis of other forms in the tissue-culture experiment (9).

The presence of coenzyme forms of B-12 bound to protein in the incubation medium indicates counter transport of converted B-12 from the interior of the cell. Cell counts done before and after incubation remained unchanged suggesting that converted B-12 does not appear in the incubation medium because of cell lysis. Schneider *et al.* (10) studied the fate of highly purified human and rabbit TC-II *in vivo*. They found that labeled TC-II disappeared rapidly from plasma due to clearance by a variety of tissues. Labeled small-molecular-weight protein fragments soon appeared in the urine. A portion of the B-12 which had been bound to the labeled TC-II was found to reenter the circulation. These *in vivo* observations are entirely consistent with our *in vitro* studies which suggest entry of the entire TC-II cyanocobalamin complex with subsequent extracellular reappearance of coenzyme forms of the vitamin.

Conclusions. In our previous experiments (4) we demonstrated that L1210 leukemic lymphoblasts were capable of taking up vitamin B-12 when bound to transcobalamin II. Intracellular B-12 was also found to be bound to a protein with the characteristics of TCII. In the present study we have demon-

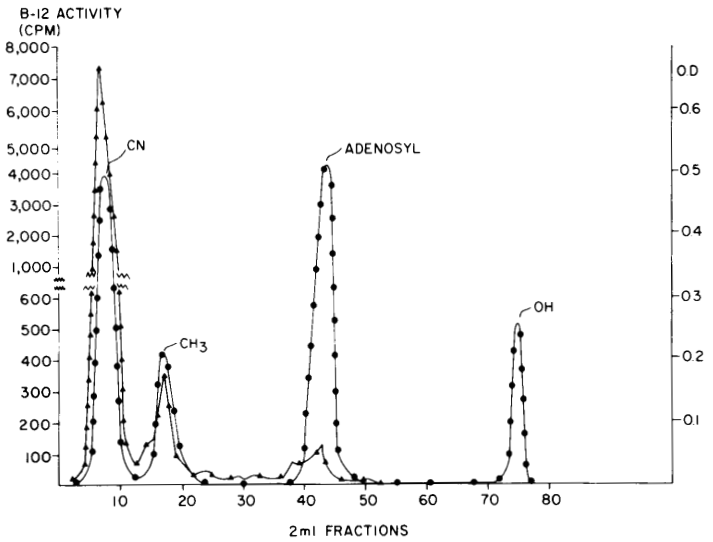

FIG. 2. Radioactivity (▲) and OD max, 365–335 nm (●) profile of the hot ethanol extract of a homogenate of L-1210 cells which had been incubated for 6 hr with TCII-bound ^{57}Co-cyanocobalamin. The extract was chromatographed with nonradioactive cobalamin standards on SP-Sephadex C-25 (column size 0.9 × 20 cm, sample size 2 ml, eluant 60 ml distilled water followed by 100 ml 0.05 *M* acetate buffer, pH 5.0. Cobalamin standards included cyanocobalamin (CN), methylcobalamin (CH_3), adenosylcobalamin (adenosyl), and hydroxycobalamin-(OH).

TABLE II. PERCENTAGE OF EACH FORM OF PROTEIN BOUND B-12 PRESENT AFTER 2 AND 6 HOURS INCUBATION WITH L-1210 CELLS.

	CN	Adenosyl	Methyl	OH
Two-hour cell supernate	94	4	2	0
Two-hour cell homogenate	94	5	1	0
Six-hour cell supernate	91	6.5	2.4	0
Six-hour cell homogenate	90	3	7	0
Six-hour plasma incubation (no cells present)	99+	0	0	0

strated that L-1210 cells do not themselves contain a TCII-like protein in the cytoplasmic soluble phase. We have further demonstrated that a portion of this protein-bound B-12 has been converted to the adenosyl and methyl forms and that such coenzyme forms may be countertransported to the exterior of the cell. These observations are consistent with our original suggestion that the entire B-12 complex crosses the plasma membrane. TCII may deliver B-12 to mitochondria where conversion to the coenzyme forms occurs. A portion of the converted vitamin appears to be transported out of the cell where it is free to recirculate.

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1. Pletsch, Q. A., and Coffey, J. W., J. Biol. Chem. **246,** 4619 (1971).
2. Newmark, P., *in* "The Cobalamins" (H. R. V. Arnstein and R. J. Wrighton, eds.), P. 79, Clowes, London (1971).
3. Toporek, M., Gizis, E. J., and Meyer, L. M., Proc. Soc. Exp. Biol. Med. **136,** 1119 (1971).

4. Ryel, E. M., Meyer, L. M., and Gams, R. A., Blood **44,** 427 (1974).
5. Lindstrand, K., and Stahlberg, K. G., Acta Med. Scand. **174,** 665 (1963).
6. Linnell, J. C., Heather, M. M., Wilson, J., and Matthews, D. M., J. Clin. Pathol. **22,** 545 (1969).
7. Tortolani, G., Biouchini, P., and Mantovani, V., J. Chromatogr. **53,** 577 (1970).
8. Peters, T. J., and Hoffbrand, A. V., Brit. J. Haematol. **19,** 369 (1970).
9. DiGirolamo, P. M., Jacobsen, D. W., and Huennekus, F. M., Amer. Soc. Hematol. (Abstr. 255), Chicago, Ill., December 1–4, 1973.
10. Schneider, R. J., Mehlman, C. S., and Allen, R. H., Amer. Soc. Hematol. (Abstr. 17), Atlanta, Ga., December 7–10, 1974.

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