

Cellular Fluxes of Vitamin B₁₂

By Fred Ostroy and Richard A. Gams

Incorporation of vitamin B₁₂ into L1210 cells requires the protein binder transcobalamin II (TCII). The process is saturable, follows Michaelis-Menten kinetics ($K_m = 2.5 \times 10^{-9} M$ at 37°C), is both temperature and calcium ($K_{50} = 1 \times 10^{-6} M$) dependent, and is inhibited by apo-TCII, indicating the presence of a TCII specific receptor on the cell membrane. B₁₂ also leaves the cell by a calcium-independent pathway bound to either TCII

or to a protein with chromatographic properties similar to those of TCIII. Since intact TCII-B₁₂ can be found in the cytosol and can promote B₁₂ uptake by mitochondria, it is proposed that the B₁₂ released from the cell bound to the TCIII-like protein is derived by mitochondrial processing of incorporated TCII-B₁₂. The slower time course of release of the latter B₁₂ is consistent with this postulate.

VITAMIN B₁₂ is carried on at least three different plasma proteins, transcobalamin (TC) I, II, and III.¹ TCI and III belong to the group of B₁₂-binding proteins known as R-binders,² a heterogeneous group of glycoproteins with similar if not identical amino acid compositions which differ primarily in carbohydrate content^{3,4} and are found in various body fluids. TCII, on the other hand, is not a glycoprotein and its molecular weight (38,000) is considerably smaller than that of R-binders.⁵

TCI does not appear to have the capacity to deliver vitamin B₁₂ to tissues. TCIII, on the other hand, promotes uptake of vitamin B₁₂ by the liver, utilizing the asialoglycoprotein pathway⁶ described by Ashwell and Morrell,⁷ and, as such, probably represents relatively nonspecific clearance. Only TCII delivers vitamin B₁₂ to a wide variety of tissues; this action has been shown both in our own and in other laboratories⁸⁻¹⁷ to occur by a highly selective calcium-dependent process.

Reports from other laboratories using L1210,¹⁶ HeLa,¹¹ or Ehrlich ascites cells^{12,17} and studies in vivo of rat liver¹⁸ and kidney¹⁹ have suggested that the delivery of vitamin B₁₂ from TCII occurs by pinocytosis. Furthermore, the studies in vivo suggest that this pinocytotic process is accompanied by lysosomal fusion with disruption of the TCII-B₁₂ complex.

Such postulated cell surface processing appears to conflict with our own observations^{8,10} that a functional TCII-B₁₂ complex can be found in the cytosol of L1210 cells and our further demonstration that TCII is required for mitochondrial uptake and subsequent coenzyme conversion of the vitamin.²⁰ These observations suggest that there is minimal alteration of the TCH-B₁₂ complex at

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Submitted March 17, 1977; accepted June 9, 1977.

Supported by USPHS Grant 5-PO2-Ca-13148 from the National Cancer Institute.

Presented in part before the American Federation for Clinical Research, New Orleans, La., January 24, 1976.

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the cell surface during incorporation. In an attempt to reconcile these differences and further our understanding of the mechanism of vitamin B₁₂ incorporation, we have reexamined the kinetics of the interaction of TCII and its cellular receptor.

MATERIALS AND METHODS

Cell Preparation

Cell preparation methods have previously been described.²¹ Briefly, L1210 cells were harvested from BDF1 mice on days 6 and 7 following intraperitoneal (i.p.) injection of 10⁵ cells. The mice were sacrificed by cervical dislocation and 5 ml of cold 20 mM Tris-saline, pH 7.5, was injected i.p. into each mouse. Cells were then expelled from the abdominal cavity after puncture with a 20-gauge hypodermic needle. The L1210 cells and some contaminating blood cells were centrifuged for 5 min at 1000 rpm (200 g) and resuspended in an equal volume of the Tris-saline buffer. Following three such washes, the remaining red cells were lysed by suspending in 0.5 volumes of 0.2% saline for 20 sec, followed by the addition of 0.5 volumes of 0.2% glucose in 1.6% saline. The cells were then washed again with the Tris-saline buffer and resuspended; the packed cell volume was determined and adjusted to between 4% and 6%. All of the above procedures were done in the cold (4°C) and cold solutions were used throughout. Trypan blue staining routinely showed that greater than 90% of the cells excluded the dye. Cells were used immediately after preparation.

Preparation of B₁₂ Binders

Except where otherwise indicated, human serum obtained from normal volunteers was used as the source of TCII. Serum was incubated with ⁵⁷Co-B₁₂ (Amersham Searle Corp., specific activity approximately 200 μCi/μg) at 37°C for 30 min, at a concentration of 1 ng/ml.

Calcium- and magnesium- "free" serum was obtained by recalcifying either fresh EDTA plasma (vacutainer 32040) or pooled acid-citrate-dextrose plasma (Fenwall TA-2) and allowing it to clot at room temperature. After removal of the clot, 100 ml of the serum was dialyzed in the cold against 4 liters of distilled water, followed by 4 liters of 1 mM EDTA, and then twice against 4 liters of 20 mM Tris-saline (pH 7.5) for 24 hr each.

⁵⁷Co-B₁₂-TCII was obtained by passing 1 ml of ⁵⁷Co-B₁₂ saturated serum through two 1.5 × 33 cm columns (connected in series) of either Sephadex G-100 or G-200. Saliva clarified by centrifugation was used as the source of R-binder. Unsaturated B₁₂-binding capacities of serum, plasma, and saliva were determined by a modification of the method of Rothenberg.²² Excess radioactive B₁₂ was added to each sample and, following a 15-min incubation at 37°C, the protein-bound B₁₂ was determined by coprecipitating with ZnSO₄ and barium hydroxide. ⁵⁷Co-B₁₂-TCII was also determined according to the method of Jacob and Herbert²³ by precipitating with Quiso G-32, generously supplied by Philadelphia Quartz Co., Valley Forge, Pa.

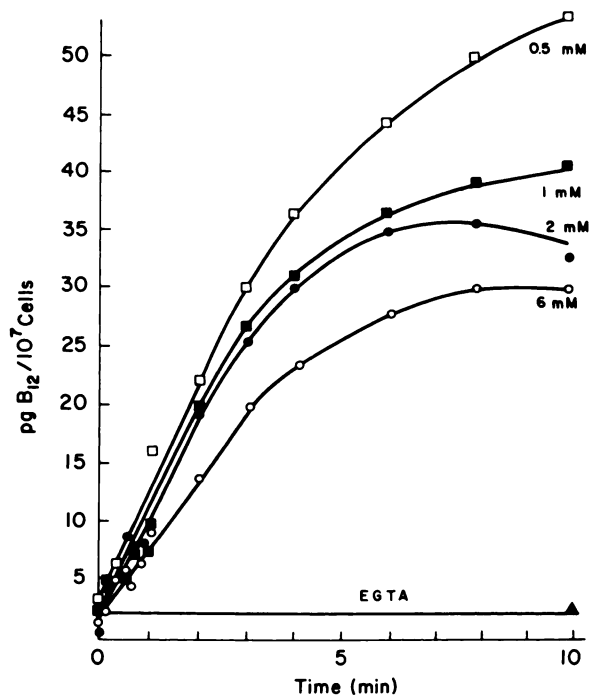
Experimental Procedures

Each milliliter of incubation mixture contained the following: 100 pg of B₁₂ (either bound or free), 0.25 ml of either 2× Eagle's minimum essential medium with spinner salts (Gibco, 78F) or 2× glucose Ringer's solution (calcium and magnesium free), or 0.5 ml of Fisher's medium for leukemic cells of mice (Gibco, 147G), and CaCl₂ or EGTA at various concentrations plus sufficient distilled water or saline to yield 0.75 ml of isotonic medium. The above media were preincubated for 5 min at 37°C prior to the addition of 0.25 volume of L1210 cells.

Assay Procedure for B₁₂ Incorporation

Millipore filters (25 mm diameter) with a pore size of 0.45 μm (Millipore Corp., HAWP-025) were soaked in 5 mM Tris-saline containing 1 mM EGTA, pH 7.0 (TSE buffer), and then were soaked for 15 min at room temperature in 5% bovine serum albumin (Sigma Chemical Co.) in TSE buffer containing 100 μg/ml crystalline vitamin B₁₂. The filters were then placed on a 30-sample manifold (Millipore), which was then evacuated. Samples (0.2 ml) of the incubation mixtures were

Fig. 1. Effect of calcium on time course of B₁₂ incorporation in L1210 cells at 37°C. Either 10 mM EGTA or calcium at the concentrations indicated was added. Incubation with an equivalent amount of salivary R-binder-⁵⁷Co-B₁₂ complex instead of serum at 1 mM CaCl₂ was also performed (Δ) with results identical to those with the addition of EGTA.



pipetted onto the filters at various time points and each filter was immediately washed three times with 2.4-ml aliquots of cold TSE buffer. Filters were counted in an automatic well scintillation counter (Nuclear Chicago model 1185) and the results, except where otherwise indicated, were the averages of duplicate determinations. The resulting counts were compared with ⁵⁷Co-B₁₂ standards of known activity. There was no significant binding of either free or protein-bound B₁₂ by the treated filters.

RESULTS

B₁₂ incorporation proceeded in a smooth continuous fashion for at least 10 min at 37°C and a typical uptake curve is shown in Fig. 1. The presence of 8 mM EGTA reduced the uptake to a low level which had no apparent time course, and which was equivalent to that found with salivary R-binder. This level is identical to the "zero" time binding found when either serum or TCII-bound B₁₂ was used. These results are consistent with both our own⁸⁻¹⁰ and other¹⁶ studies showing that only TCII can promote uptake in L1210 cells and that it does so by a calcium-dependent process. The latter point is further substantiated by the data presented in Fig. 2A, showing that EGTA could progressively inhibit B₁₂ uptake in the presence of excess (5 mM) magnesium and that this inhibition could be reversed by the addition of calcium (Fig. 2B). From these data, a *K*₅₀ for calcium was calculated to be 0.9×10^{-6} M using the EGTA binding constants for the various ions present.²⁴ Despite the necessity of calcium for B₁₂ uptake, levels at or above 1 mM tended to reduce the maximal level of cell-associated B₁₂, though there was minimal effect on initial velocity of incorporation (see Fig. 6).

The effect of TCII-B₁₂ concentrations on incorporation into L1210 cells is

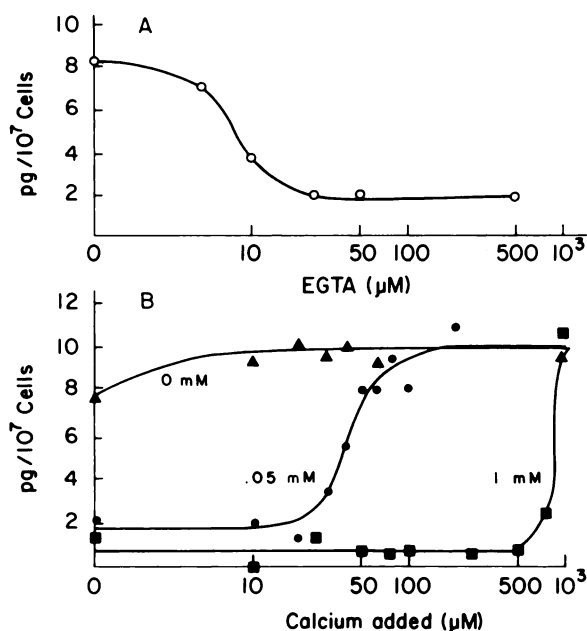


Fig. 2. Effect of EGTA and calcium on B₁₂ incorporation determined by using dialyzed serum and (A) EGTA added as indicated or (B) calcium added in the presence of no EGTA, 50 μM EGTA, or 1 mM EGTA as indicated. Media contained 5 mM magnesium.

shown in Fig. 3. The reciprocal plot of these data (inset of Fig. 3) demonstrates that Michaelis-Menten type kinetics were followed with V_{max} of 0.18 pmoles/min/10⁻⁷ cells and a K_m of 2.5×10^{-9} M at 37°C. Increasing levels of serum containing apo-TCII and R-binder progressively inhibited the B₁₂ uptake by cells incubated with serum whose B₁₂-binding capacity had been saturated, whereas a 20-fold excess of unsaturated salivary R-binder had no effect. Anal-

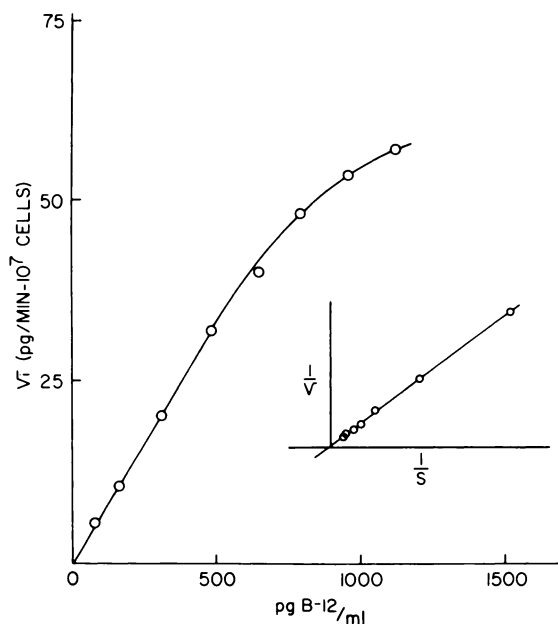


Fig. 3. Initial velocities of B₁₂ incorporation determined from slopes of curves obtained by sampling at 25, 35, 60, 90 and 120 sec after addition of various concentrations of TCII-B₁₂ to cell suspension at 37°C. Inset shows data replotted as reciprocals.

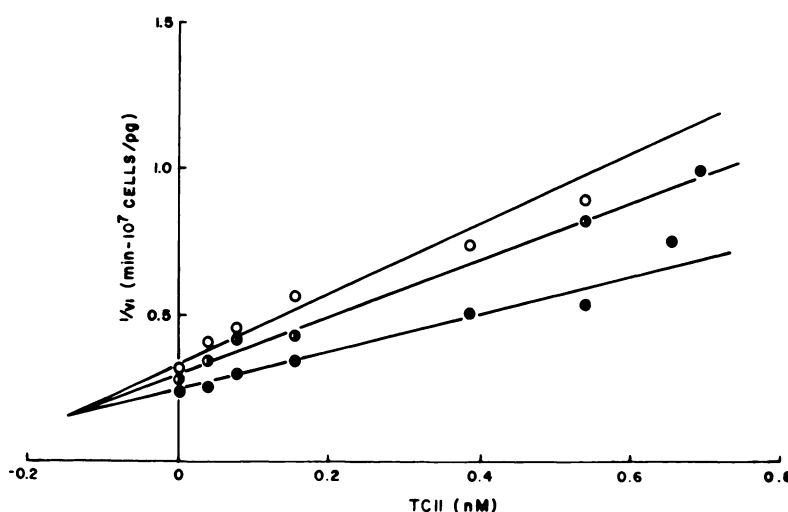


Fig. 4. Effect of apo-TCII on initial velocity (V_i) of B₁₂ incorporation. Incubation was performed at 25°C and contained 100 (○), 200 (◻), or 400 (●) pg/ml of TCII-B₁₂ and indicated level of apo-TCII. Data are presented as a Dixon plot and demonstrate competitive inhibition.

ysis of the initial velocities of incorporation determined at 25°C at several levels of TCII and TCII-B₁₂ using the direct linear plot method of Eisenthal and Cornish-Bowden²⁵ suggests that apo-TCII is a competitive inhibitor of TCII-B₁₂ uptake. The data are shown as a Dixon plot in Fig. 4. These results are in agreement with the observations by Carmel and Bjorndal²⁶ and of Friedman et al.²⁷ that both apo- and holo-TCII are bound by red cells and human placental membranes, respectively, and explain the observation¹¹ that optimal B₁₂ incorporation into Ehrlich ascites cells occurs when the binding capacity of ascites fluid is saturated.

The effect of temperature on the B₁₂ incorporation process was also investigated and the early phase of incorporation is shown in Fig. 5. At 25° and 37°C incorporation continued until a maximum level was reached which was inversely related to temperature. At 4°C, B₁₂ incorporation continued in a linear fashion for at least 4 hr, eventually reaching a level greater than the maximum incorporation obtained at either 25° or 37°C. The initial velocity of incorporation, on the other hand, exhibited a direct dependence on temperature and was largely unaffected by the presence of calcium in excess of 1 mM (Fig. 6). EGTA inhibited uptake at all temperatures.

Whereas the maximal level of B₁₂ incorporation was inversely related to both temperature and higher levels of calcium (Fig. 1), the initial velocity was relatively independent of calcium at these levels and directly dependent on temperature. These findings suggest that more than one process may be going on simultaneously. One such process may be B₁₂ efflux from the cells. In order to test this hypothesis L1210 cells were incubated with ⁵⁷Co-B₁₂-TCII for 10 min and then washed three times with ice-cold saline and reincubated in a serum-free medium (Fig. 7). During the 20 min that the washing procedure required, little or no radioactivity was lost. When the cells were reincubated at 37°C

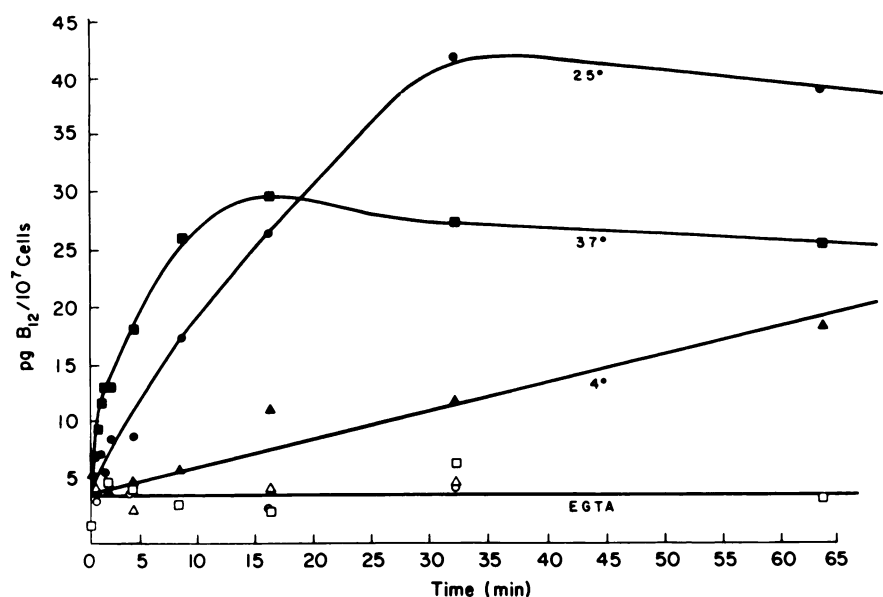


Fig. 5. Effect of temperature on time course of B_{12} uptake with calcium concentration at 1 mM and temperature as indicated. Open symbols indicate the presence of 10 mM EGTA. Though not shown, incorporation at 4°C continued in a linear fashion for at least 4 hr.

there was a prompt and nearly complete loss of accumulated B_{12} . The initial rate of loss did not appear to be calcium dependent, and at least some of the material released may have been reincorporated in the presence of 0.5 mM calcium. The effect of temperature on release is shown in Fig. 8. Though there was little loss of label at 4°C, approximately 75% of the incorporated vitamin was

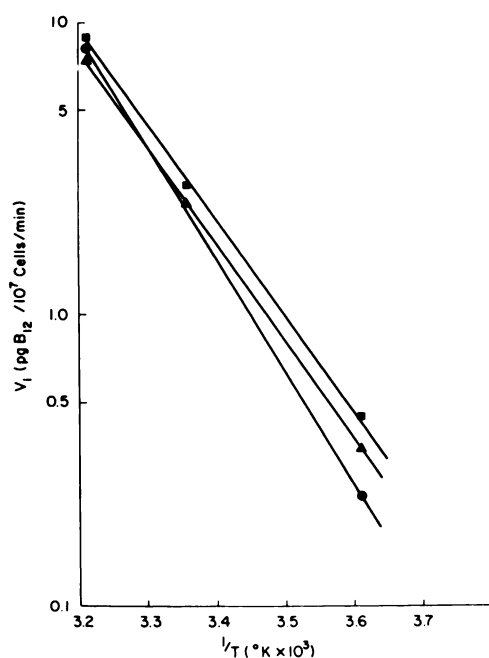
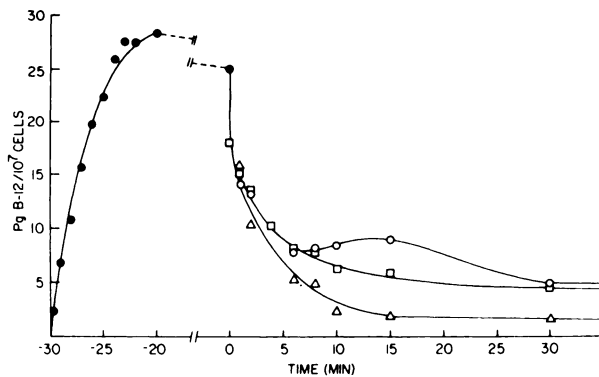


Fig. 6. Initial velocities (V_i) of B_{12} uptake determined from time courses of B_{12} uptake as described in Fig. 3 at calcium concentrations of 1 (●), 2 (▲), and 5 mM (■).

Fig. 7. Effect of calcium ions on release of incorporated B₁₂ determined by incubating cells in medium containing 100 pg/ml of ⁵⁷Co-B₁₂ (serum bound) and 0.5 mM calcium (●) for 10 min followed by washing in cold TSE buffer. After washing, cells were resuspended in media containing either 5 mM calcium (□), 0.5 mM calcium (○), or 5 mM EGTA (Δ) and incubated at 37°C.



lost within 15 min at 37°C. When the incubation medium was replaced by medium containing radioactive B₁₂ instead of the nonradioactive vitamin, continued incorporation of B₁₂ was evident. In contrast to the effect of temperature, the presence or absence of either serum or calcium had little or no effect on efflux.

In order to determine the nature of the material being released, cells were first incubated with TCII-B₁₂ obtained by Sephadex G-100 chromatography of serum saturated with radioactive B₁₂ (Fig. 9A). The medium in which the cells were incubated was then rechromatographed on the same column. With EGTA present in the incubation mix, less than 3% of the total B₁₂ present was retained and no alteration of the TCII-B₁₂ complex could be seen (Fig. 9B). With calcium present, the cells retained 17% of the B₁₂ present while 33% (24% of the total) of the B₁₂ found in the medium was bound to a species with a molecular weight similar to that of the R-binders (Fig. 9C). Identical results have also

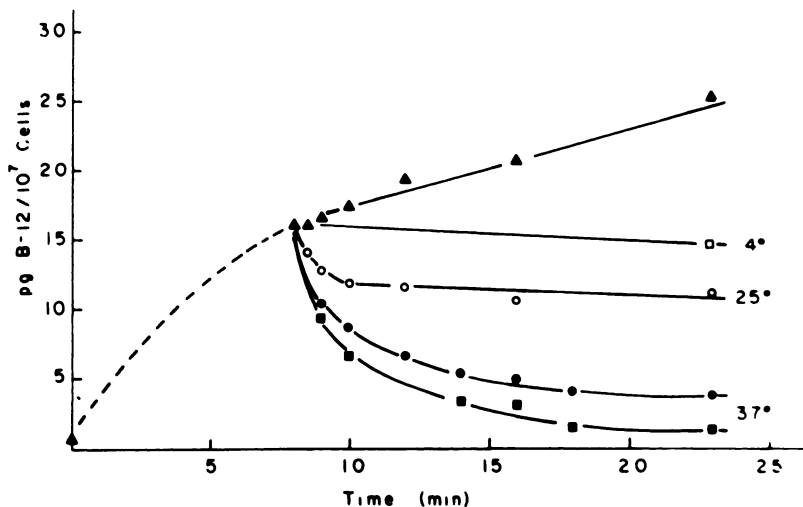


Fig. 8. Effect of temperature on release of incorporated B₁₂ determined as in Fig. 7, except that cells were reincubated in media containing 100 pg of nonradioactive serum-bound B₁₂ and 0.5 mM calcium at temperatures indicated. In addition, some contained serum-bound ⁵⁷Co-B₁₂ in place of the nonradioactive isotope at 37°C (▲) and some contained 8 mM EGTA (■).

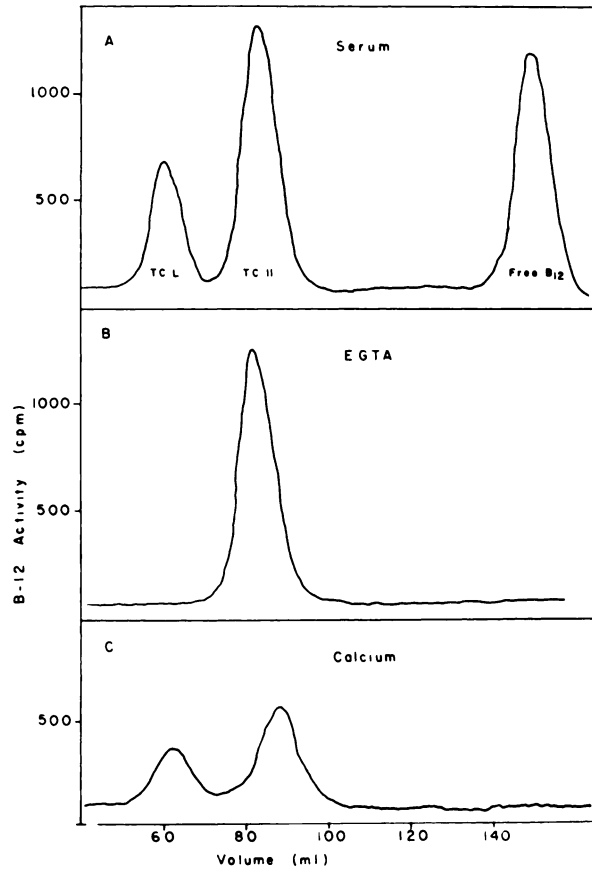


Fig. 9. Sephadex G-100 filtration of B_{12} -binding proteins obtained from the following: (A) Serum saturated with $^{57}\text{Co}-B_{12}$. The three peaks represent, in order of increasing elution volume, TCI (large molecular weight binder), TCII, and free (unbound) B_{12} . (B) The TCII peak, isolated in A, following incubation with L1210 cells for 1 hr at 37°C in the presence of 10 mM EGTA. Cells were removed by centrifugation at 200 g for 5 min. (C) The TCII peak, isolated in A, following incubation with L1210 cells for 1 hr at 37°C in the presence of 1 mM CaCl_2 .

been obtained with unfractionated serum. The time course of release of B_{12} associated with the larger molecular weight protein was determined by pre-incubating cells with $^{57}\text{Co}-B_{12}$, washing, and then reincubating in media containing nonradioactive B_{12} . At various intervals aliquots of the incubation mix were taken and, after the cells were removed, the TCII in the medium was adsorbed onto Quso G-32, a specific TCII binder,²³ and the non-TCII-associated

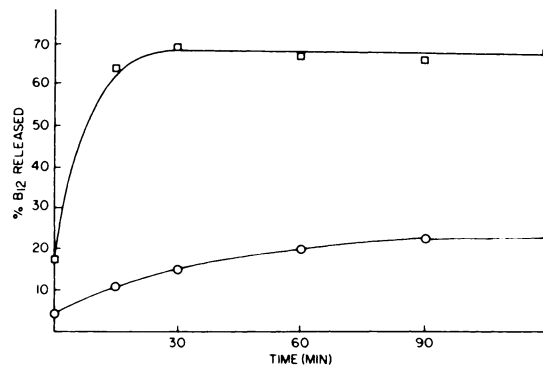


Fig. 10. Time course of release of TCII- B_{12} (□) and TCI- B_{12} (○) as determined by Quso G32 adsorption. Results are expressed as percentage of B_{12} incorporated following a 10-min incubation at 37°C . Prior to reincubation at 37°C , cells were washed three times in the cold with TSE buffer.

B₁₂ was determined (Fig. 10). Most of the B₁₂ released within the first 15 min was bound to TCII (Quso-adsorbed material); however, 27% of the B₁₂ was released more slowly. None of this material was lost by overnight dialysis, and it was eluted from DEAE at the same salt concentration as TCIII.²⁸

DISCUSSION

The data presented in this paper show that L1210 cells incorporated TCII-bound B₁₂ by a calcium- ($K_{50} = 0.9 \times 10^{-6} M$), TCII-, and temperature-dependent process. Unlike the biphasic time course of B₁₂ incorporation in L1210 cells reported by DiGiralomo and Huennekins,¹⁶ we found that uptake proceeded in a smooth continuous fashion for at least 10 min and was qualitatively similar to the time course reported for HeLa cells¹³ and more recently reported for Ehrlich ascites cells¹⁷ and human fibroblasts.¹⁵ Following an initial period of increasing B₁₂ incorporation, which was inversely related to temperature, the level of cell-associated vitamin began to decline slowly. The maximum level of incorporation obtained was also inversely related to temperature (Fig. 5) and to calcium (Fig. 1) and suggests that an ongoing efflux process occurs simultaneously with influx. When preloaded cells were allowed to reincubate in fresh medium, both TCII-B₁₂ and a protein-B₁₂ complex similar to TCIII-B₁₂ could be found in the external medium. In contrast to the uptake process, release of TCII-B₁₂ was not calcium dependent and had a somewhat different temperature coefficient, as demonstrated by its nearly complete inhibition at 4°C (Figs. 7 and 8) despite a significant rate of uptake at this temperature (Fig. 5). Both of these properties suggest that the mechanism of release is not exchange of surface-bound B₁₂ but rather that at least two distinct processes occur. The continued incorporation of B₁₂ following a change of medium (Fig. 8) is consistent with this conclusion since simple exchange would not be accompanied by a net increase in uptake.

In view of our published reports that TCII-B₁₂ can be found in the cytosol⁸ and that TCII-B₁₂ promotes mitochondrial uptake of B₁₂,²⁰ we feel that the above data indicate that TCII-B₁₂ is incorporated into the cell by a process distinct from pinocytosis with lysosomal fusion. Pierce et al.¹⁷ using Ehrlich ascites cells, also reported a smooth continuous uptake curve and a rapid release of TCII-B₁₂ from cells incubated with TCII-B₁₂. They proposed, however, that TCII-B₁₂ release is due to surface exchange and that the B₁₂ not released is sequestered into micropinosomes or similar sequestra on the cell surface membrane. Subsequent incorporation into the cell may then occur through the mediation of an intracellular binder, but without lysosomal fusion. This conclusion is in part based upon their *in vivo* studies showing that after B₁₂ is incorporated into Ehrlich ascites cells it is found to be associated with a large molecular weight binder and that no such binder can be found on lysing the cells following *in vitro* studies.

We also have reported that only TCII-B₁₂ is present in the soluble phase of L1210 cells.⁸ We have now demonstrated, however, that there is a second component of B₁₂ release from L1210 cells and that it consists of B₁₂ associated with a protein with chromatographic properties similar to TCIII. While it is possible that the TCII found in the cytosol and released following reincubation is lo-

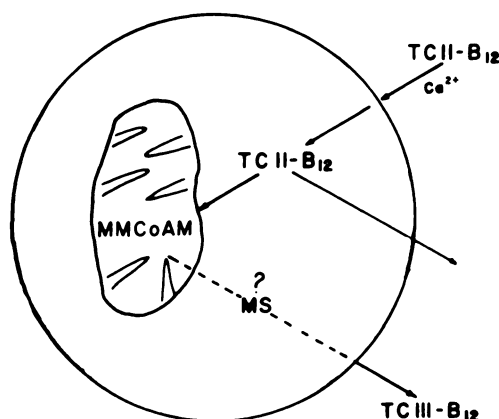


Fig. 11. Possible model for B₁₂ incorporation. Following its inclusion into the cell, TCII-B₁₂ can move out of the cell by a calcium-independent process or can bind to the mitochondria, where the B₁₂ can be utilized by the mitochondrial enzyme, methylmalonyl coenzyme A mutase (MMCoAM). After mitochondrial utilization, B₁₂ is released from the cell on a new binder chromatographically similar to TCIII. The role that the B₁₂-requiring enzyme methionine synthetase (MS) plays in the release process, if any, is still uncertain.

cated entirely on the surface of the cells, a more likely explanation is that the entire TCII-B₁₂ complex is incorporated into the cells and then into the mitochondria, where the B₁₂ is transferred to a TCIII-like protein and released. In addition to the slower time course of release, several other observations are also consistent with the latter hypothesis. No unsaturated B₁₂-binding proteins have been found in the cell cytosol in either L1210 cells,¹⁰ Ehrlich ascites cells,¹⁷ or human fibroblasts,²⁹ despite attempts to find such binders. However, they are apparently present in mitochondria.³⁰ A correlation between the amount of B₁₂ converted to the coenzyme forms and the level of large molecular weight binding protein has been reported.¹⁷ Finally, a recent report by Kolhouse and Allen³¹ indicates that the large molecular weight B₁₂-binding proteins found in human fibroblasts are the B₁₂-dependent enzymes, methylmalonyl coenzyme A mutase (MMCoAM) and methionine synthetase (MS), and that the incorporated B₁₂ appears on the mitochondrial enzyme MMCoAM prior to its appearance on the enzyme MS.

In conclusion, we feel that the model presented in Fig. 11 is a reasonable explanation of our data and is not inconsistent with the recent data obtained by others using Ehrlich ascites cells and human fibroblasts. TCII-B₁₂ enters the cell intact, thereby precluding pinocytosis with lysosomal fusion as the method of incorporation. Once in the cell, the TCII-B₁₂ can then either leak out or be incorporated into mitochondria. Following or coincident with mitochondrial uptake, B₁₂ is converted to the adenosyl form and bound to the B₁₂-dependent enzyme MMCoAM. Once mitochondrial utilization has occurred, the B₁₂ is released and eventually exits the cell bound to a TCIII-like protein. The relationship between this protein and the two enzymes, MMCoAM and MS, is not clear and will require further investigation.

ACKNOWLEDGMENT

We wish to express our sincere thanks to Dr. Frank Schabel of the Southern Research Institute for providing us with BDF1 mice harboring L1210 leukemic cells, to Dr. Eoline I. McGowan of the Birmingham Veterans Administration Hospital for providing us with acid-citrate-dextrose plasma, and to Mary Sabens for her technical assistance during the course of these studies.

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