

Protein-mediated Uptake of Vitamin B₁₂ by Isolated Mitochondria

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Protein-mediated B₁₂ uptake by isolated rat liver mitochondria has been shown to be enhanced by plasma transcobalamin (TC-II) but not by salivary R binder in vitro. The process is enhanced by calcium and depends on active mitochondrial respiration. Following uptake, cyanocobalamin is converted to adenosyl and methylcobalamins and released from the mitochondria. TC-II appears to be required for both cellular and mitochondrial uptake of vitamin B₁₂.

OF THE RECOGNIZED BINDERS of vitamin B₁₂, only gastric intrinsic factor (IF) and plasma transcobalamin II (TC-II) enhance specific cellular uptake of the vitamin. There is evidence in a number of systems that the entire protein B₁₂ complex may enter the cell.

Pletsch and Coffey¹ studied the uptake of vitamin B₁₂ by rat liver in vivo after the intracardiac injection of radiolabeled vitamin. Intracellular B₁₂ was found to be associated with a protein having the same molecular weight as TC-II. Newmark² also found that intracellular TC-II bound B₁₂ in the fractionated rat kidney. Toporek et al.³ measured the hepatic uptake of vitamin B₁₂ mediated by hog IF. The vitamin was subsequently transferred to bile which facilitated absorption of vitamin B₁₂ in a second perfused liver. This observation suggested cellular uptake of a protein B₁₂ complex in view of the fact that the bile per se did not enhance hepatic uptake of B₁₂.

We have previously investigated the uptake of vitamin B₁₂ from plasma employing an in vitro system of isolated murine L1210 leukemic lymphoblasts.⁴ The majority of intracellular B₁₂ was found in the cytoplasmic soluble phase bound to a protein having the physical properties of TC-II and the capacity to deliver B₁₂ to fresh L1210 cells. This observation was felt to be consistent with the possibility that the entire TC-II B₁₂ complex had entered the cell. A significant fraction of intracellular B₁₂ was also found in the mitochondrial fraction. Peters and Hoffbrand⁵ and Rothenberg⁶ have also found B₁₂ associated with mitochondria in the guinea pig ileal enterocyte. In Pletsch and Coffey's study,¹ B₁₂ accumulated in the mitochondrial fraction. It seemed possible, therefore, that after entering the cell, TC-II delivered B₁₂ to mitochondria for conversion to coenzyme forms. To investigate this possibility we have studied the uptake of vitamin B₁₂ by isolated mitochondria.

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MATERIALS AND METHODS

Cell Fractionation

Male Sprague-Dawley rats were killed by cervical dislocation. The livers were removed, patted dry on filter paper, weighed, and placed in a pH 7.3 solution of sucrose (0.25 *M*), Tris-HCl (1 *mM*), ethylene-diaminetetraacetate (EDTA, 1 *mM*), and bovine serum albumin (1%). This medium was designated "STEА." Following removal of the liver all procedures were carried out at 4°C. The tissue was minced and transferred to a Dounce glass homogenizer containing 10 ml STEА/g of tissue. The tissue was then homogenized with a motor-driven Teflon pestle to obtain an even suspension. The homogenate was then centrifuged at 600 *g* for 10 min and the supernatant solution was filtered through cheese cloth. This suspension was then subjected either to zonal centrifugation or differential centrifugation.

Zonal Centrifugation

The zonal fractionation technique has been described in detail previously.^{4,7} Briefly, a discontinuous sucrose density gradient in Tris-buffered saline consisting of 55 ml of 9%, 70 ml of 25%, 80 ml of 35%, and 100 ml each of 43%, 47%, and 50% sucrose with a 55% sucrose cushion was loaded into a spinning (3000 rpm) Ti-14 Spinco zonal rotor in order of increasing density. The homogenized sample was layered over the gradient and separated from the rotor core by a 60-ml overlay of buffer. The system was maintained at 4°C. After an 11-min centrifugation at 45,000 rpm, the gradient containing the fractionated components was displaced from the spinning (3000 rpm) rotor with 57% sucrose. The effluent was continuously monitored at 280 nm on a Beckman DB-GT spectrophotometer and collected in 20-ml fractions. These fractions were analyzed for sucrose concentration which was determined on a Bausch and Lomb Abbe 3-L refractometer. Each fraction was assayed for succinate dehydrogenase (SDH)⁸ to serve as a marker for fractions rich in mitochondria, and 5'-nucleotidase activity⁹ as a marker for fractions rich in plasma membranes. Peak fractions were then assayed for the ability to take up vitamin B₁₂ from serum as described below under experimental procedures.

Preparation of Mitochondria by Differential Centrifugation

In all other experiments, rat liver mitochondria were prepared employing the method described by Sordahl and Schwartz¹⁰ for normal and tumor mitochondria. After obtaining the tissue homogenate as described above, the suspension was centrifuged at 8500 *g* for 12 min. The mitochondrial pellet was then washed three times with fresh STEА, centrifuging each time at 8500 *g* for 10 min., and finally suspended in STEА, 0.5 ml/g of original starting material. Final mitochondrial suspensions contained 20-30 mg protein/ml. Tightly coupled oxidative phosphorylation was demonstrated for each mitochondrial preparation employing a Clarke polarographic electrode. Representative preparations were examined by electron microscopy (Fig. 1).

Vitamin B₁₂ Binders

Human serum and saliva were obtained from the same healthy donor. Samples were stored at -20°C. Recalcified plasma was subjected to gel filtration on Sephadex G-200 to separate the large molecular weight transcobalamins (TC-I, TC-III) from transcobalamin II (TC-II).

Experimental Procedures

The unsaturated B₁₂ binding capacity (UBBC) of each sample was determined by adding an excess of ⁵⁷Co-B₁₂ (Amersham/Searle Corp., Arlington Heights, Ill.; S.A. 50-150 mCi/mg) followed by exhaustive dialysis against normal saline or gel filtration on Sephadex G-25. Saliva (UBBC, 26 ng/ml) was diluted with normal saline to approximate the binding capacity of human serum (UBBC, 1.5 ng/ml). Incubation mixtures consisted of 500 μl STEА (made 1 *mM* with CaCl₂), 50 μl of B₁₂ binder (human serum, TC-I, TC-II, saliva, or normal saline), and 50 pg of ⁵⁷Co-B₁₂ delivered in 50 μl of normal saline. The amount of B₁₂ added represented approximately two-thirds of the UBBC of the serum or diluted saliva. To each of these mixtures was added 50 μl of mitochondrial suspension, or 100 μl of each zonal fraction. Samples were incubated at 25°C in a Brinkman Thermostat (model 3401, Brinkman Instruments, Westbury, N.Y.) followed by centrifugation for 1 min in a Brinkman Microcentrifuge (model 3200). The pellets were washed

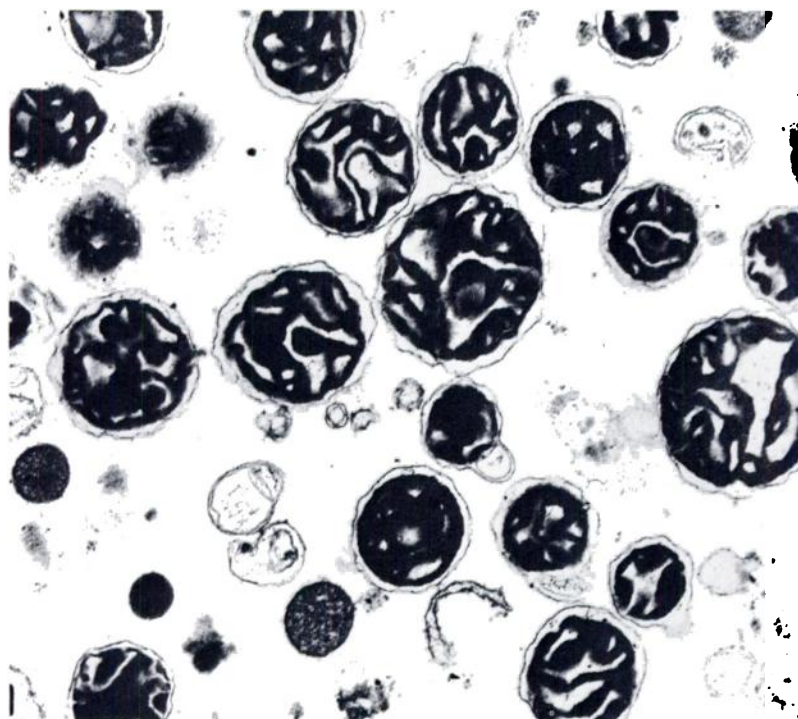


Fig. 1. Electron micrograph of mitochondrial pellet prepared by differential centrifugation, fixed immediately at 4°C in 3% glutaraldehyde buffered with 0.1 mM pH 7.4 cacodylate. Specimens were then postfixed in 2% osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were stained with uranyl acetate-lead citrate and examined in a Hitachi Perkin-Elmer HU-11C electron microscope. The preparations were found to contain intact mitochondria, some mitochondrial fragments, and a small amount of material which has the appearance of plasma membrane vesicles. $\times 7500$.

three times with 1 ml STEA and the radioactivity retained by the mitochondrial pellet determined in an automatic well scintillation counter (Nuclear Chicago Model 1185) calibrated with ⁵⁷Co-B₁₂ samples of known activity.

Additional uptake experiments were performed in the presence of 10^{-2} M CaCl₂, 5×10^{-4} M KCN, 5×10^{-3} M barbital, 5×10^{-3} M atractylasate, and 5×10^{-3} M 2,4-dinitrophenol.

Extraction and Separation of Cobalamins

A 2-ml mitochondrial suspension was incubated at 37°C for 2 hr with 2 ml of recalcified human plasma, diluted saliva, or normal saline containing 2 ng of ⁵⁷Co-cyano-B₁₂. The sample was then centrifuged at 8500 g for 15 min, and the mitochondrial pellet was suspended in 4 ml deionized water and sonicated. Cobalamins were then extracted from the supernatants and sonicates by the hot ethanol procedure described by Lindstrand and Stahlberg¹¹ as modified by Linnel et al.¹² In brief, each sample was brought to 20 ml with deionized water, 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 was 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 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.¹³ 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, San Diego, Calif.), and hydroxycobalamin (Sigma Chemical Corp., St. Louis, Mo.) 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. All incubation, extraction, and separation procedures were carried out in the dark or with a red safelight to prevent photolytic degradation of B₁₂ analogues.

RESULTS AND DISCUSSION

We have previously demonstrated that L1210 leukemic lymphoblasts took up vitamin B₁₂ when it was bound to plasma TC-II.⁴ Subcellular fractionation indicated that the majority of the vitamin B₁₂ was in the cytoplasmic soluble phase bound to a protein with physical and functional characteristics of TC-II, with the next largest amount associated with a mitochondrial fraction. We subsequently demonstrated¹⁴ that L1210 cells did not contain a cytoplasmic endogenous B₁₂-binding protein. This finding strongly suggested that the TC-II B₁₂ complex had entered the cell intact. In the present study, we investigated the possibility that, once inside the cell, TC-II delivered B₁₂ to mitochondria.

Uptake of Vitamin B₁₂ by Mitochondria

The results of zonal fractionation of liver cell homogenate are shown in Fig. 2. Those fractions (V and VI) richest in SDH activity, the mitochondrial

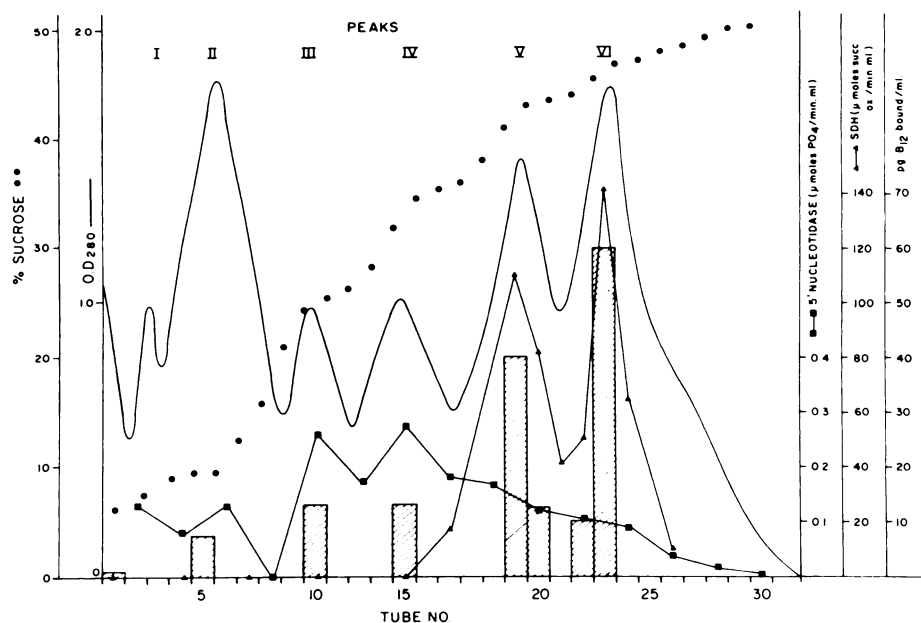


Fig. 2. Zonal profile of whole liver homogenate containing six banding zones in order of increasing density showing 5'-nucleotidase activity, SDH activity, and B₁₂ uptake (bar diagrams) of peak fractions. Each peak corresponds to one step in the discontinuous gradient.

Table 1. ⁵⁷Co-B₁₂ Uptake by Isolated Mitochondria*

⁵⁷ Co-B ₁₂ Carrier	Additions	Uptake (pg/mg Protein)
Serum (human)	0	14.3
Serum (human)	EGTA (10 mM)	0.9
Saliva	0	0.8
Saline	0	1.1
Saline	Serum† (human)	1.8
Saliva	Serum† (human)	0.9
Serum (human)	Saliva†	14.3
Serum (human)	Serum† (human)	8.1
Serum (rat)‡	0	20.0

*Prepared by differential centrifugation.

†Previously saturated with nonradioactive B₁₂ and containing the same B₁₂ concentration as the ⁵⁷Co-B₁₂ carrier.

‡Obtained from male Sprague-Dawley rats.

marker, took up the greatest amount of vitamin B₁₂ from serum. Significant binding was also noted in peaks III and IV which contained the highest 5'-nucleotidase activity, the plasma membrane marker. From the amount of B₁₂ binding noted in peaks III and IV, however, it was clear that plasma membrane contamination could not account for the binding seen in the mitochondrial fractions. Although not shown in Fig. 2, the addition of 10 mM EGTA virtually eliminated B₁₂ uptake in all fractions, suggesting calcium dependence of B₁₂ uptake from serum in both the mitochondrial and plasma membrane fractions.

The results of incubation of isolated mitochondrial suspensions with ⁵⁷Co-B₁₂ bound to serum, salivary R binder, or the free vitamin in saline are shown in Table 1, and the uptake from separated serum binders is shown in Table 2. Significant uptake occurred only when mitochondria were incubated with serum (Table 1) or the TC-II fraction of serum (Table 2) carrying ⁵⁷Co-B₁₂. Uptake did not occur when the mitochondria were incubated with either free radioactive B₁₂, with the labeled vitamin bound to the salivary R binder, or the TC-I fraction of serum. Further, uptake by these fractions could not be induced by the addition of serum previously saturated with nonradioactive B₁₂, demonstrating the absence of other plasma factors promoting uptake. The addition of saliva saturated with nonradioactive B₁₂ did not diminish serum-mediated uptake of radioactive vitamin, suggesting that salivary R binder did not compete with the active serum fraction for mitochondrial uptake. The addition of

Table 2. Mitochondrial Uptake of ⁵⁷Co-B₁₂ From Separated Serum Binders*

⁵⁷ Co-B ₁₂ Carrier	Addition	Uptake (%)
TC-II†	0	10
TC-II	EGTA (10 mM)	2
TC-I‡	0	1
TC-I	EGTA (10 mM)	0.7

*Mitochondria prepared by differential centrifugation.

†Containing 15 pg ⁵⁷Co-B₁₂.

‡Containing 30 pg ⁵⁷Co-B₁₂. TC-I implies the larger molecular weight B₁₂-binding Sephadex fraction.

equal quantities of serum previously saturated with nonradioactive B₁₂ to the serum containing ⁵⁷Co-B₁₂ significantly diminished uptake, demonstrating competition for mitochondrial receptors. These data are taken to be consistent with the hypothesis that the uptake of vitamin B₁₂ by mitochondria, as in intact cells, is mediated by TC-II. Rothenberg⁶ fractionated ileal enterocytes following incubation with radioactive B₁₂ bound to intrinsic factor. He found vitamin B₁₂ bound to an intrinsic factor-like protein in the mitochondrial fraction. This observation in ileal cells would be analogous to the present suggestion that the transport protein, whether it be intrinsic factor or TC-II, crosses the plasma membrane to deliver vitamin B₁₂ to mitochondria.

Pletsch and Coffey¹ investigated the uptake of vitamin B₁₂ in vivo following intracardiac injection of ⁵⁷Co-B₁₂ in the rat. They found radiolabeled vitamin in the liver associated with a protein having the same molecular weight as TC-II. Furthermore, ⁵⁷Co-B₁₂ was found to accumulate with time in the mitochondrial fraction. These authors suggested that the TC-II B₁₂ complex had entered the cell following which TC-II was destroyed perhaps by lysosomes releasing the vitamin B₁₂ for subcellular distribution. They admitted, however, that it was not possible to determine whether all or part of the newly absorbed B₁₂ passed through the lysosomes before accumulating in the mitochondrial or soluble fractions.

The data presented in the present investigation would suggest that TC-II is required for mitochondrial uptake of vitamin B₁₂. Since it has been clearly demonstrated that asialoglycoproteins may be nonspecifically taken up by hepatocytes, probably by pinocytosis,¹⁵ it may be that the TC-II B₁₂ complex enters the liver cell by two mechanisms. Some of the TC-II B₁₂ is taken up nonspecifically by pinocytosis and is destined to destruction in the lysosomal fraction. Burger et al.²² have recently shown that TC-III and the granulocyte B₁₂ binding protein are normally cleared by the hepatic asialoglycoprotein clearance mechanism. Another portion may pass into the hepatocyte soluble fraction from which the vitamin is delivered to mitochondria. Our previous studies with L-1210 cells⁴ have clearly demonstrated a cytoplasmic soluble fraction of B₁₂ bound to a protein with the characteristics of TC-II.

The addition of 10 mM EGTA to serum almost completely inhibits uptake of vitamin B₁₂. This observation strongly suggests that mitochondrial uptake of the vitamin is calcium dependent. This finding would be analogous to the calcium requirement for the uptake of vitamin B₁₂ from TC-II or IF in intact cell systems^{4,16,18} as well as the calcium dependence for B₁₂ uptake in nonfractionated liver homogenates.¹⁹

The influence of metabolic inhibitors²⁰ on the mitochondrial uptake of B₁₂ is shown in Table 3. Atractylasate, a drug which prevents the flux of adenine nucleotides into mitochondria, had virtually no effect on the uptake of B₁₂. Barbital and cyanide which inhibit 50%-70% of mitochondrial respiration significantly diminished the B₁₂ uptake. 2,4-Dinitrophenol, which uncouples oxidative phosphorylation, had no effect. Mitochondrial uptake of B₁₂ thus appears to depend on active respiration but is independent of the influx of adenine nucleotides or the maintenance of tightly coupled oxidative phosphorylation.

Table 3. Effect of Metabolic Inhibitors on ⁵⁷Co-B₁₂ Uptake by Isolated Mitochondrial Suspensions*

Inhibitor	Uptake (pg/mg Protein)
Control	14.3
Atractylasate	15.3
Barbital	7.9
Cyanide	7.4
2,4-Dinitrophenol	14.5

*Prepared by differential centrifugation.

Mitochondrial Conversion of Cyanocobalamin

Mitochondrial suspensions were incubated with radiolabeled cyanocobalamin bound to plasma, saliva, or as the free vitamin in saline. Following incubation, the cobalamins were extracted and separated by SP-Sephadex C-25 chromatography as previously described with intact L-1210 cells.¹¹ The percentages of the various cobalamins present after such incubation are shown in Table 4. The only significant conversion of cyanocobalamin occurred when mitochondria were incubated with vitamin bound to plasma transcobalamin II. From 8% to 18% conversion occurred in several experiments. Approximately equal amounts of adenosylcobalamin and methylcobalamin were produced. In no instances were detectable levels of hydroxycobalamin encountered. In all experiments, significantly greater amounts of coenzyme forms of B₁₂ were found in the incubation supernatants than in the mitochondrial sonicates, suggesting that after conversion to coenzyme forms, the majority of the converted B₁₂ was released from the mitochondria. This finding would be consistent with our previous observations¹⁴ of counter transport of converted forms of B₁₂ from intact L1210 cells. Furthermore, Schneider et al.²¹ have employed in vivo methods to study the fate of purified human and rabbit TC-II. The protein was cleared rapidly from the plasma, but the labeled B₁₂ bound to it reappeared in the circulation.

The results of the present study are consistent with our original suggestion that the entire B₁₂ TC-II complex crosses the plasma membrane. TC-II then delivers B₁₂ to mitochondria where conversion to various coenzyme forms

Table 4. Conversion of Cyanocobalamin to Other Forms by Isolated Mitochondrial Suspensions*

Media†	Cyano (%)	Adenosyl (%)	Methyl (%)	Hydroxy (%)
Plasma—supernate pellet	82	10	8	0
	96	2	2	0
Saliva—supernate pellet	100	0	0	0
	100	0	0	0
Saline—supernate pellet	99	Trace	Trace	0
	100	0	0	0
Plasma control‡	100	0	0	0

*Prepared by differential centrifugation.

†All contain 10 mM CaCl₂.

‡Incubated in the absence of mitochondria.

occurs. A portion of the converted vitamin is then released from mitochondria to be transported out of the cell where it is free to recirculate.

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