JOURNAL OF BACTERIOLOGY, June 1971, p. 745-750
Vol. 106, No. 3

Printed in U.S.A.

Transport of Vitamin B_{12} in *Escherichia coli*[1]

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Received for publication 31 December 1970

The uptake of ^{60}Co-labeled cyanocobalamin (vitamin B_{12}) by cells of *Escherichia coli* K-12λ was shown to consist of an initial rapid phase (complete in <1 min), followed by a slower secondary phase. Methods enabling the measurement of ^{60}Co-B_{12} uptake after incubation times of 1 to 2 sec were used in studies on the initial rate of B_{12} uptake. This initial process showed saturation kinetics, with a V_{max} of 56 molecules per sec per cell and a K_m of 5 nm, and was essentially independent of cellular energy metabolism. No inhibition was obtained with cyanide, fluoride, arsenite, or 2,4-dinitrophenol, and an energy of activation of 3.8 kcal/mole for this initial phase of uptake was calculated from its response to temperature changes between 15 and 35 C. The inhibition by $HgCl_2$ (50% at 0.1 mM) but not by 1 mM *N*-ethylmaleimide or 1 mM *p*-chloromercuribenzoate was consistent with a role for a relatively inaccessible sulfhydryl residue at the initial B_{12} binding site. The secondary phase of B_{12} uptake was clearly dependent on the energy metabolism of the cell, and, from its response to temperature, an energy of activation of about 17 kcal/mole was calculated. Cyanide (10 mM), arsenite (10 mM), and 2,4-dinitrophenol (0.1 mM) gave at least 70% inhibition of the rate of the secondary phase. The formation of other cobalamins, such as 5'-deoxyadenosyl cobalamin, was not an obligate part of B_{12} transport. The cells were also able to take up ^{60}Co-labeled cobinamide cyanide.

Biological transport processes have received much experimental attention in recent years, much of which has been paid to the uptake of macronutrients, such as sugars, amino acids, and various inorganic ions. However, we believe that the study of micronutrient transport has some experimental advantages. Although the required amounts of the micronutrients are small, the amounts normally available to the cells are correspondingly low. Accordingly, at least as far as vitamin B_{12} transport is concerned, cells have apparently developed very powerful transport systems for micronutrients, enabling them to compete effectively for the small amounts available. In the microbial transport of vitamin B_{12}, this effectiveness consists of an initial B_{12}-binding system with a very high affinity for vitamin B_{12}, coupled with large B_{12} storage capacities. *Ochromonas malhamenis* (*unpublished data*) and *Lactobacillus leichmannii* (5) are able to store 10^3 to 10^4 times their minimal requirement of vitamin B_{12}. Our primary interest is in the microbial transport of vitamin B_{12}, and this paper reports on the basic properties of B_{12} transport in *Escherichia coli*, a system which has been largely neglected since the early work of Oginsky (6).

[1] A preliminary report of this work was presented at the 70th Annual Meeting of the American Society for Microbiology in Boston, Mass., 26 April-1 May 1970.

MATERIALS AND METHODS

Organism. A culture of *Escherichia coli* K-12λ was obtained from J. W. Ogilvie and was maintained at room temperature on nutrient agar.

Cobalamin compounds. 5'-Deoxyadenosyl cobalamin, DBC, and methyl cobalamin, CH_3-B_{12}, were obtained from Pierrel, Milan, Italy. Cyanocobalamin, CN-B_{12}, and aquocobalamin, HO-B_{12}, were provided by the Sigma Chemical Co. and Mann Research Laboratories, respectively; E. R. Squibb & Sons, New York, supplied the ^{60}Co-labeled cyanocobalamin of specific activity 0.85 to 1.7 mCi/μmole. ^{60}Co-labeled cobinamide cyanide, Factor B, was prepared from ^{60}Co-CN-B_{12} by the method of Friedrich and Bernhauer (3).

Growth of E. coli. The cells used in the experiments were grown aerobically at 37 C, on the minimal medium of Davis and Mingioli (1), supplemented with 0.5% glucose and 100 μg of thiamine per liter. The growth of the cultures was monitored by measuring the optical density (OD) at 660 nm with a Beckman DU-2 spectrophotometer, and the cells were usually harvested during the late log or early stationary phases. The cells were washed once with, and then suspended in, 0.1 M potassium phosphate (*p*H 6.6) containing 1% glucose. In some experiments, the cell number was determined with a Coulter Counter. A cell suspension of 10^9 cells per ml gave an OD at 660 nm of about 0.8.

Assay of ^{60}Co-B_{12} uptake. The general method used was to preincubate the cells in the phosphate-glucose buffer under given conditions for about 15 to 30 min before addition of the ^{60}Co-B_{12}, usually to give a final concentration of 2 to 6 nM. After various time periods, portions were removed, and filtered through Millipore filters (25-mm diameter, 0.45-μm pore size) to separate the cells from the reaction mixtures. The filters were washed with 10 ml of buffer at the incubation temperature, dried, and counted in a Beckman model LS-233 liquid scintillation counter. Blanks were determined in all experiments by carrying out the same procedures in the absence of cells. Two specialized methods were developed for measuring the uptake of ^{60}Co-B_{12} after very short incubations.

Flow uptake method. This method was used for incubations from 1.5 to about 15 sec, and was carried out at 25 C. The cells were preincubated in a reaction mixture which contained 0.1 M phosphate-glucose buffer (*p*H 6.6) and a suspension of Hyflo Super Cel (a diatomaceous filter aid). Portions of this reaction mixture containing 10^8 to 5×10^8 cells and 25 mg of Hyflo Super Cel were gently sucked down onto a membrane filter (Millipore Corp.) under vacuum (about 5 inches of Hg) from a Gast vacuum pump. The Super Cel served both as a filter aid and as a support for the cells. Immediately after the buffer was sucked through the thin layer of cells and Super Cel, a portion of buffer containing ^{60}Co-CN-B_{12} was delivered from an automatic pipette and allowed to flow through the cell layer. The transit time of this solution through the cell layer was timed with a stopwatch and was taken as the incubation time of the cells with the labeled B_{12}. This technique enabled measurements to be made after incubation times as short as 1 sec. Immediately after exposure to the ^{60}Co-B_{12}, the cell layer was washed with 10 ml of buffer without B_{12}. The membrane filters, together with the cell layer, were then dried and counted. We considered the possibility of consistent errors being introduced by the retention of appreciable amounts of ^{60}Co-CN-B_{12} in the filter pads of Hyflo Super Cel, with continued uptake by the cells, during the period of 1 sec or so between the incubation and the wash. Errors of this sort would be expected to give plots in which zero uptake would not coincide with zero time. Extrapolation of the experimental results gave zero uptake intercepts which were usually within 0.25 sec of zero time. Accordingly, we have concluded that the errors in timing the incubations were usually not more than about 0.25 sec.

Rapid dilution method. This method was derived from that of Kaback (4) and was usually used for incubation times of 5 and 10 sec. Portions (0.95 ml) of a preincubation reaction mixture were transferred to 20-ml test tubes and stirred magnetically; buffer (50 μliters) containing ^{60}Co-CN-B_{12} was then injected from a syringe fitted with a Hamilton repeating dispenser. After an appropriate incubation time interval, usually 5 sec, the sample was rapidly diluted with 10 volumes of buffer, delivered from an automatic pipette. The diluent buffer contained unlabeled CN-B_{12} at about 10 to 20 times the concentration of the ^{60}Co-CN-B_{12} in the reaction samples. The diluted reaction mixtures were filtered through membrane filters, which were then washed with 10 ml of buffer without B_{12}, dried, and counted. Three to five replicates were usually done for all experiments using the rapid dilution or flow assay methods.

RESULTS

Time course of B_{12} uptake. Figure 1 shows a typical time course of B_{12} uptake when cells of *E. coli* were incubated at 32 C with 2.3 nM ^{60}Co-CN-B_{12}. There was a very rapid initial phase of uptake, which was complete within 1 min; this was followed by a slower secondary phase, which may be linear for 20 to 30 min, until finally a plateau was reached. In this experiment, when a 400-fold excess of unlabeled CN-B_{12} was added to the reaction mixture, there was no release of ^{60}Co from the cells, indicating that the ^{60}Co-CN-B_{12} taken up by the cells was not readily exchangeable with that in the medium. In other experiments, under similar conditions, occasionally as much as 5% of the B_{12} taken up by the cells was readily exchangeable. The rate of the initial phase of B_{12} uptake was usually about 5 to 10 times that of the secondary phase. Each of these phases has been studied separately.

Initial phase of B_{12} uptake. Figure 2 shows that the initial uptake of ^{60}Co-CN-B_{12} was linear for only 8 to 10 sec. Thereafter, the rate fell rapidly until the rate of the secondary uptake was obtained. Incubations of 5 sec or less were routinely used in experiments in which an estimate of the initial rate of uptake was desired. When the membrane filters with the cells were washed with buffer containing high concentrations of unlabeled CN-B_{12} (100 to 400$\times$ the ^{60}Co-CN-B_{12} concentration in the reaction mixtures), less than 5% of the ^{60}Co-B_{12} was lost from the cells, showing that the B_{12} taken up by the cells during the initial phase was not readily exchangeable with that in the medium. The *p*H optimum of the initial rate of B_{12} uptake was 6 to 6.6 (Fig. 3), with a very sharp drop in activity at *p*H values below the optimum.

The initial phase of vitamin B_{12} uptake shows saturation kinetics, and the effects of variations in the concentration of CN-B_{12} upon the rate of this process are shown in Fig. 4. The data were analyzed by using the nonlinear regression method of Wilkinson (9), and the rate was found to be half-maximal at about 5 nM CN-B_{12}. The line for the inset double reciprocal plot in Fig. 4 was drawn from the K_m and V_{max} values given by the Wilkinson analysis. The cell number was not counted in this experiment, but the results from a similar experiment, in which the cell number was known, gave a V_{max} for the initial uptake of vitamin B_{12} of 56 molecules per sec per cell.

The initial rate of B_{12} uptake was relatively

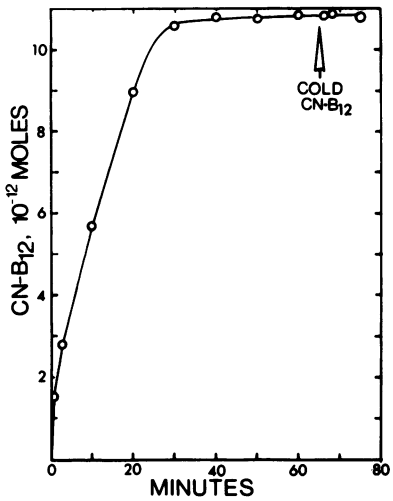

FIG. 1. *Time course of uptake of ^{60}Co-CN-B_{12} by cells of E. coli. Reaction conditions: 32 C, aerobic, 50 mM potassium phosphate (pH 6.6), 0.5% glucose, 0.2% Hyflo Super Cel, and 2.3 nM ^{60}Co-CN-B_{12}. The cell density would have given an OD at 660 nm of 0.15. There was a 15-min preincubation before addition of ^{60}Co-B_{12}; 4-ml samples were taken. Unlabeled CN-B_{12}, to give a final concentration of 0.9 μM, was added at 65 min (arrow).*

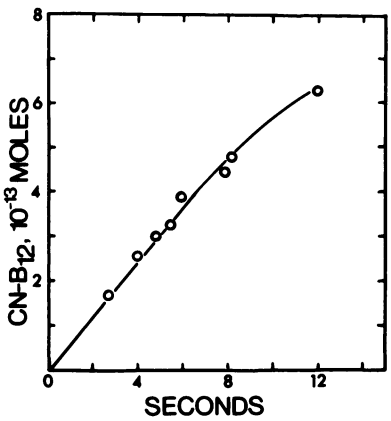

FIG. 2. *Linearity of the initial uptake of ^{60}Co-CN-B_{12} by cells of E. coli. Reaction conditions: 25 C, aerobic, 50 mM potassium phosphate (pH 6.6), 0.2% glucose, 0.5% Hyflo Super Cel, 1.47 nM ^{60}Co-CN-B_{12}. The amount of cells used would have given an OD at 660 nm of 0.4. Portions (2 ml) were removed for assay by the flow method.*

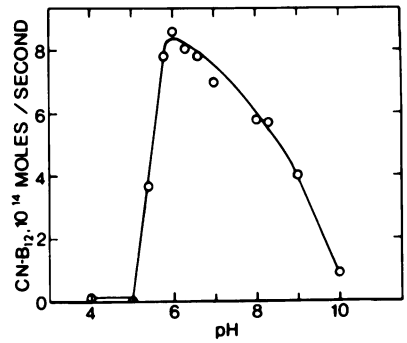

FIG. 3. *Effects of pH on the initial rate of ^{60}Co-CN-B_{12} uptake by cells of E. coli. Reaction conditions: 25 C, aerobic, buffer containing 50 mM sodium glutamate, histidine-hydrochloride, and potassium phosphate adjusted to the appropriate pH with KOH, 1% glucose, and 0.4% Hyflo Super Cel. The density of the cells in the reaction mixture would have given an OD at 660 nm of 0.47. Preincubations were 15 to 30 min; 1-ml portions were removed for assay by the flow method. ^{60}Co-CN-B_{12} concentration was 3.72 nM.*

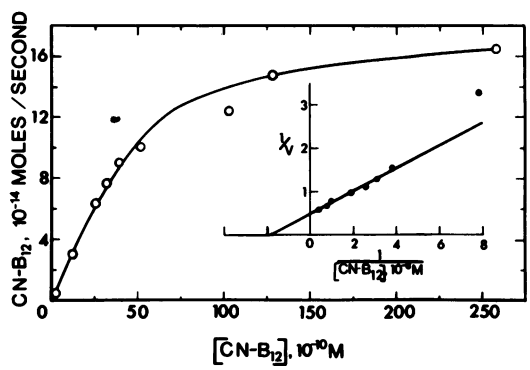

FIG. 4. *Effects of CN-B_{12} concentration upon the initial rate of ^{60}Co-CN-B_{12} uptake by cells of E. coli. Reaction conditions: 25 C, aerobic, flow assay method with various ^{60}CN-B_{12} concentrations. The cells (OD_{660} 0.4) were preincubated in 0.1 M potassium phosphate (pH 6.6) with a 0.4% Hyflo Super Cel. Samples (1 ml) were used. The inset shows a Lineweaver-Burk plot of the same data. v = CN-B_{12} taken up, 10^{-13} moles per sec.*

insensitive to changes in temperature and to the presence of cellular poisons. There was no inhibition when the following compounds, at the stated concentrations, were included in the reaction mixtures: 10 mM KCN, 0.2 mM 2,4-dinitrophenol, 0.1 mM arsenite, 10 mM sodium fluoride, 1 mM *N*-ethylmaleimide, and 1 mM *p*-chloromercuribenzoate. The only compound tested so far which was inhibitory was $HgCl_2$, which gave 50% inhibition at about 0.1 mM. The effects of temperature on the initial uptake of vitamin B_{12} are shown in Fig. 5. The rate of this process showed a Q_{10} of about 1.2 below the optimum of 35 C, giving an energy of activation of about 3.8 kcal/mole, which was calculated by means of the Arrhenius equation.

Secondary phase of B_{12} uptake. The rate of the secondary uptake of ^{60}Co-CN-B_{12} was usually measured by determining the increment in ^{60}Co-

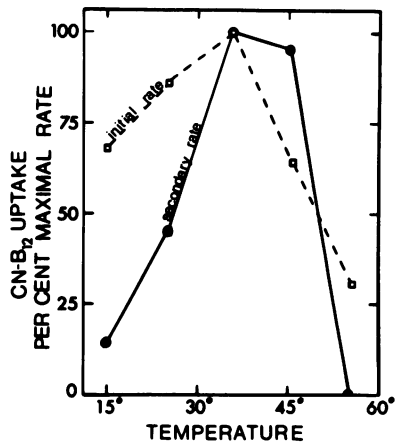

FIG. 5. *Effects of temperature upon the initial and secondary rates of ^{60}Co-CN-B_{12} uptake by cells of E. coli. Initial uptake: (□) cells (0.4 OD_{660}) in 0.1 M potassium phosphate (pH 6.6) plus 0.4% Hyflo Super Cel, rapid dilution method, 10-sec incubation, 5.1 nM ^{60}Co-CN-B_{12}. Secondary uptake: (●) cells (0.4 OD_{660}) in 0.1 M potassium phosphate (pH 6.6) plus 1% glucose, 5.1 nM ^{60}Co-CN-B_{12}, 1-ml samples. ^{60}Co-B_{12} uptake was measured between 2 and 20 min after addition of the ^{60}Co to the reaction mixtures. The results are expressed as a per cent of the maximum rate.*

B_{12} uptake between 3 and 10 min after addition of the labeled B_{12} to the reaction mixture. This part of the B_{12} transport process differed from the initial uptake in that it was found to be clearly dependent on the energy metabolism of the cell. The effects of temperature on the rate of this process are shown in Fig. 5, and gave an average Q_{10} of about 2.7 in the range from 15 to 35 C and an optimum between 35 and 40 C. The activation energy was calculated to be in the range of about 17 kcal/mole. Figure 6 shows the effects of glucose and of a variety of metabolic inhibitors upon the secondary uptake of ^{60}Co-CN-B_{12} by cells which had been stored overnight at 5 C. Substantial inhibition was given by 10 mM cyanide, 10 mM arsenite, and 0.2 mM 2,4-dinitrophenol, but 10 mM fluoride was inhibitory only in the absence of glucose.

The secondary uptake of vitamin B_{12} was usually accompanied by the conversion of the ^{60}Co-CN-B_{12} into other cobalamins, primarily DBC and HO-B_{12} with lesser amounts of CH_3B_{12} (data not shown, methods as in reference 2). It was thought that such a conversion may be an obligate part of the energy-dependent component of B_{12} transport. In some experiments, however, active secondary uptake has also been observed without significant conversion of the CN-B_{12} into other cobalamins. We have concluded that the B_{12} transport process in *E. coli* does not necessarily involve the formation of other cobalamins.

Specificity of the B_{12} transport process. Cobinamide cyanide, Factor B, labeled with ^{60}Co, was prepared from ^{60}Co-CN-B_{12} and taken up by cells of *E. coli* (Fig. 7). After correcting for the different concentrations used in this experiment, a comparison of the relative rates of uptake of CN-B_{12} and Factor B indicated that the initial uptake process was probably unable to distin-

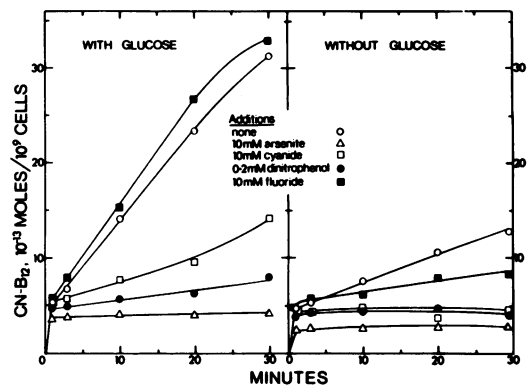

FIG. 6. *Effects of glucose and of some metabolic inhibitors upon the uptake of ^{60}Co-CN-B_{12} by cells of E. coli. Reaction conditions: 37 C, aerobic, 0.1 M potassium phosphate (pH 6.6), 4 nM ^{60}Co-CN-B_{12}, 10^9 cells/2-ml sample. The cells had been stored overnight at 5 C and were preincubated for 30 min before the addition of the labeled CN-B_{12}. Other additions: none, ○; 10 mM sodium arsenite, △; 10 mM KCN, □; 0.2 mM 2,4-dinitrophenol, ●; 10 mM sodium fluoride, ■. Right, without glucose; left, with 1% glucose.*

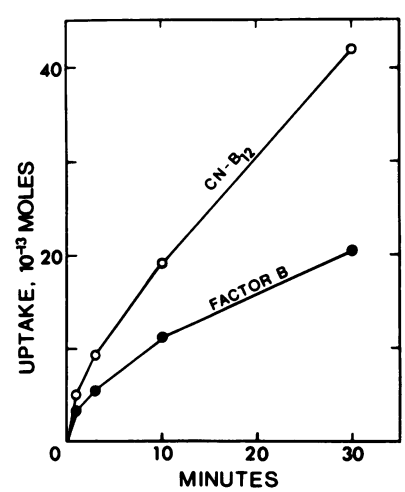

FIG. 7. *Uptake of ^{60}Co-CN-B_{12} and of ^{60}Co-cobinamide cyanide, Factor B, by cells of E. coli. Reaction conditions: 30 C, aerobic, 50 mM potassium phosphate (pH 6.7), 0.5% glucose, cell density at an OD of 660 nm was 0.06 and either 3 nM ^{60}Co-CN-B_{12} or 2.1 nM Factor B. Preincubation was 20 min; 4-ml samples were taken.*

guish between these compounds, but the secondary rate of uptake of Factor B was significantly less than that of CN-B_{12}.

DISCUSSION

Wild-type *E. coli* neither synthesizes nor requires vitamin B_{12}, but it possesses an effective transport system for this compound. This transport system resembles those previously described for the protozoan, *O. malhamensis* (8), and Ehrlich ascites cells (7) in that it is a biphasic process, consisting of an initial rapid phase which is essentially independent of the energy metabolism of the cell, followed by a slower, energy-dependent secondary phase. The ascitic system differs significantly in that the B_{12} must first become bound to a protein in the ascitic fluid, and it is this protein-B_{12} complex which is the molecular species taken up by the ascites cells. No evidence for the participation of an extracellular B_{12} binding protein has been found for B_{12} transport in *E. coli*.

By means of assay methods which can measure the uptake of vitamin B_{12} after incubation periods as short as 1 to 2 sec, we were able to measure the initial rates of B_{12} uptake under various experimental conditions. The initial phase of B_{12} uptake shows saturation kinetics, with a V_{max} of about 56 B_{12} molecules per sec per cell, and the rate is half-maximal at about 5 nM CN-B_{12}, indicating that the B_{12} binding site on the cell surface has a very high affinity for vitamin B_{12}. We believe that this initial process may represent the loading, with B_{12}, of a membrane-bound B_{12} carrier. Consistent with this view are the observations that this initial phase of B_{12} uptake is largely insensitive to changes in temperature from 15 to 35 C, and to the presence of a variety of inhibitors of the energy metabolism of the cell. The inhibition by mercuric chloride, but not by *N*-ethyl maleimide or *p*-chloromercuribenzoate, may be an indication that a relatively inaccessible sulfhydryl residue is necessary for the initial binding of vitamin B_{12}. Ca^{2+} has been found to stimulate the uptake of CN-B_{12} in *O. malhamensis* at supraoptimal *p*H values (8). Preliminary results (*data not shown*) indicate that in *E. coli* the decrease in the rate of the initial uptake of CN-B_{12} at *p*H values below the optimum can be partially prevented by mM $CaCl_2$.

The slower secondary phase of B_{12} uptake may represent the energy-dependent release of the B_{12} from a membrane binding site into the interior of the cell. The energy dependence of this phase is reflected in its sensitivity to changes in temperature, with an activation energy of about 17 kcal/mole, compared with 3.8 kcal/mole for the initial phase, and to the presence of metabolic inhibitors such as 2,4-dinitrophenol, arsenite, and potassium cyanide. This energy-dependent phase of B_{12} uptake does not result in a detectable chemical modification of the CN-B_{12}, but the possibility of a transient change cannot be ruled out.

The specificity of the B_{12} transport system in *E. coli* has not been widely studied, but this organism is capable of taking up cobinamide cyanide and thus differs from B_{12} transport in *O. malhamensis* (Arch. Biochem. Biophys., *in press*). We found less than 5% of the ^{60}Co-CN-B_{12} taken up by the cells at any stage was readily exchangeable with vitamin B_{12} in the medium. Similarly, Oginsky found relatively little exchange of CN-B_{12} when the cells were incubated in the presence of glucose, but the amount of CN-B_{12} which was readily exchangeable with that in the medium was much greater in the absence of glucose (6).

It is possible that the two phases of vitamin B_{12} transport in *E. coli* represent two distinct processes, but we believe that they are sequential parts of a single process. Although an active initial phase of uptake can be observed in the absence of a secondary uptake under various experimental conditions, the reverse has never been observed. The following paper (2), which describes the isolation and properties of B_{12} transport mutants from *E. coli*, provides further evidence in support of this conclusion.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the expert technical assistance of Yvette A. Preston.

This investigation was supported by Public Health Service research grant AM12653 from the National Institute of Arthritis and Metabolic Diseases.

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