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Further Studies on the Binding of Vitamin B_{12} to the Cell Wall of a B_{12}-Requiring *Lactobacillus*

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The vitamin B_{12}-binding property of *Lactobacillus leichmannii* ATCC 7830 has been studied. The organism could bind 0.52 μg of B_{12} per mg of cells. With regard to the cellular site for B_{12} accumulation, three-quarters of the B_{12} bound to the cell was found in the crude cell wall fraction, and the remaining one-quarter was found in the particulate (ribosome) fraction. After receiving enzymatic treatments with ribonuclease, lipase, and trypsin, the wall fraction retained three-fifths of the initial B_{12}. The possibility of cross-contamination of the wall and particulate fractions was excluded by measuring the contents of ribonucleic acid and hexosamines in each fraction. The B_{12}-binding activity of the wall was destroyed by pretreatment of the wall with pepsin, Pronase, or trypsin. However, once bound to the wall, the B_{12} was not released by the same treatments. These facts suggest that B_{12} is bound to a polypeptide in the wall on which these enzymes act and that, once bound, B_{12} somehow inhibits the enzymatic actions as described earlier with *L. delbrueckii* no. 1. A B_{12}-polypeptide complex was isolated by treatment with 0.2 N HCl from walls to which B_{12} had been bound. The complex was then purified. The complex moves as a single band on polyacrylamide gel electrophoresis. Its molecular weight was estimated around 21,500 with microheterogeneity on a Sephadex G-75 column. The mode of B_{12} binding was found to be similar to that of *L. delbrueckii*.

We have previously shown (13) that vitamin B_{12}-requiring lactobacilli characteristically take up and preserve large amounts of B_{12} in cells from surrounding media. This property was analyzed by using *Lactobacillus delbrueckii* no. 1, a B_{12}-requiring strain. The organism takes up about 8×10^4 molecules of B_{12} per cell, 130- to 160-fold as many as those required for normal cell proliferation of this organism. The accumulation of B_{12} by the cell occurs whether the organism is growing or resting with no exogenous energy supply, and the accumulated B_{12} can be utilized for subsequent growth. The cellular site of B_{12} accumulation was shown to be located principally in the cell wall where a B_{12}-binding principle exists (17-19). A B_{12} complex was isolated from the wall and analyzed for its nature (20, 21). On the other hand, Beck and his co-workers, who studied the binding of B_{12} to the cell of *L. leichmannii* ATCC, 7830, stated that B_{12} is bound almost exclusively in the ribosomes (8, 9). On the basis of the similarities in taxonomical characteristics of *L. leichmannii* and *L. delbrueckii* (12, 16), we suggested (17, 18) that B_{12} may be bound first to the wall in *L. leichmannii* as well; the suggestion was supported by Kinoshita et al. (10, 11) with *L. leichmannii* ATCC 4797. However, they did not perform a quantitative analysis of B_{12} distribution within the cell, i.e., what percentage and amount of B_{12} is bound to the wall (maximum binding capacity of the isolated wall) as we did with *L. delbrueckii* no. 1 (17, 18).

This paper describes the results of such studies with *L. leichmannii* ATCC 7830 and some properties of a B_{12} binder in its wall which are somewhat different from those of *L. delbrueckii* no. 1. B_{12} complexes (B_{12} binders to which B_{12} is bound) were isolated from the wall and ribosomes of *L. leichmannii* by treatment with 0.2 N HCl and purified. A comparative study was also made for B_{12}-binding capacities of cells and isolated walls of several strains belonging to *L. delbrueckii* and related species.

MATERIALS AND METHODS

Cultivation of organism. *L. leichmannii* ATCC 7830 was mainly used. The organism was grown stationary at 37 C in inoculation medium for *L. leichmannii* (Nissui Seiyaku Co., Tokyo, Japan), harvested by centrifugation at the early stationary phase of growth, washed twice with 10 mM tris(hydroxymethyl)aminomethane (Tris)-succinate buffer, *p*H 7.2, containing 5 mM magnesium acetate (TSM), and subjected to further studies. When the B_{12}-binding capacity of the whole cell was examined, cells were washed once with distilled water instead of TSM.

Fractionation of cellular components. Washed cells of *L. leichmannii* were suspended in TSM and disrupted by treatment either with a sonic oscillator (10 kc) as described previously (18) or with a French pressure cell. With this organism, the two methods of disruption gave somewhat different yields for the wall (sedimentable at 27,600 × *g* for 20 min) and particulate (sedimentable at 107,000 × *g* for 120 min) fractions; therefore, the method employed is specified in each experimental section. Cellular components were separated by differential centrifugation as follows. Walls were sedimented at 27,600 × *g* for 20 min and washed twice with TSM. The supernatant solution and washings from the walls were combined and again subjected to centrifugation at the same speed for 20 min. The precipitate obtained was omitted from the present study. The supernatant solution was centrifuged at 107,000 × *g* for 120 min, resulting in its separation into the particulate (ribosomal) and soluble fractions.

Other organisms which were used in the experiments summarized in Table 1 were disrupted by passing through a French pressure cell; walls were obtained as with *L. leichmannii*.

All the fractionation procedures were carried out at 0 to 4 C.

Treatments of walls with enzymes or HCl, or both. Methods are described below for small-scale experiments which were conducted to examine the effect of treatments with various enzymes and HCl solutions on the B_{12}-binding activity of the wall of *L. leichmannii*.

To investigate the distribution of B_{12} within the cell of *L. leichmannii*, cells which had bound B_{12} were disrupted sonically and fractionated as above. The crude wall preparation received enzymatic treatments with ribonuclease and lipase under the conditions described below, except that the relative concentrations of ribonuclease and lipase to the preparation were approximately 7 and 2.5 times higher, respectively, and the volume of reaction mixture was small. After these treatments, trypsin (about 0.1 mg per mg of walls) was added to the above mixture, which was then incubated with stirring overnight at 37 C. In every enzymatic treatment, drops of toluene were added as a preservative. The treated walls were washed three times with TSM (RLT-wall). To obtain a B_{12} complex from the wall, the walls which were washed with TSM as above were washed once more with 0.05 M phosphate buffer (*p*H 7.0) and suspended in the same buffer at an approximate concentration of 25 mg of walls/ml in a final volume of 150 ml. After addition of 5 mg of B_{12}, the suspension was incubated with stirring for 20 min. The suspension then was added to 5 mg of ribonuclease and 100 mg of lipase and was incubated with stirring for 24 hr at 37 C. Drops of toluene were added as above. After the incubation the walls were washed twice with distilled water by centrifugation (RL-wall) and suspended in distilled water. HCl was added to the suspension to give a concentration of 0.2 N and an approximate RL-wall concentration of 15 mg per ml. The suspension then was incubated with stirring for 42 hr at 37 C. The B_{12} was found as a B_{12} complex in the supernatant solution after this treatment. The residual wall fraction was sedimented by centriguation and washed with a small volume of distilled water (RLH-wall); the washing was combined with the above supernatant solution. The yield of the RLH-wall was about 68% of that of the RL-wall. In other words, 32% of the latter fraction was solubilized into the supernatant solution in which the B_{12} complex was contained.

Electron microscopy. Electron micrographs (Fig. 1) of whole cells, crude walls, and enzymatically treated walls of *L. leichmannii* were taken on carbon-coated preparations with a Hitachi HU-11 DS electron microscope (Tokyo).

Determination of chemical marker substances. Total sugars were estimated by the anthrone method (24) with glucose as standard. Ribonucleic acid (RNA) was estimated by the method of Ceriotti (3) with yeast RNA (Sigma Chemical Co., St. Louis, Mo.) as standard. Total hexosamines were estimated by the method of Boas (2), with glucosamine hydrochloride as standard, after hydrolysis of the cellular component with 3 N HCl in a sealed tube at 105 C for 20 hr and subsequent evaporation of HCl.

Determination of B_{12}. Vitamin B_{12} was determined either by bioassay with *L. leichmannii* ATCC 7830 by using B_{12} assay medium USP (Difco Laboratories, Detroit, Mich.) or from radioactivity by using ^{60}Co-labeled B_{12}, which was counted with a well scintillation counter. The concentration of a standard solution of B_{12} was determined from its optical density at 361 nm.

Purification of B_{12} complex. Purification of a B_{12} complex, released from the RL-walls by treatment with 0.2 N HCl, was carried out as follows. The solution of the 0.2 N HCl treatment and the washing from the treated walls were combined and neutralized with 10 N NaOH. White precipitates which appeared upon neutralization were removed by centrifugation. The supernatant solution was dialyzed in a cellophane bag against 0.01 M phosphate buffer, *p*H 7.0, containing 0.1 M NaCl (PBN) at 4 C for 48 hr. Then, the dialyzed material was placed onto a CM-cellulose column equilibrated with the same solution. The column was eluted first with PBN and then with a linear gradient concentration of phosphate buffer, *p*H 7.0, containing a constant concentration of 0.1 M NaCl. Subsequent to the purification process, optical densities at 280 and 361 nm were measured as described previously (20).

Gel filtration. Sephadex G-75 (Pharmacia, Uppsala, Sweden) in PBN at 4 C was used with a column

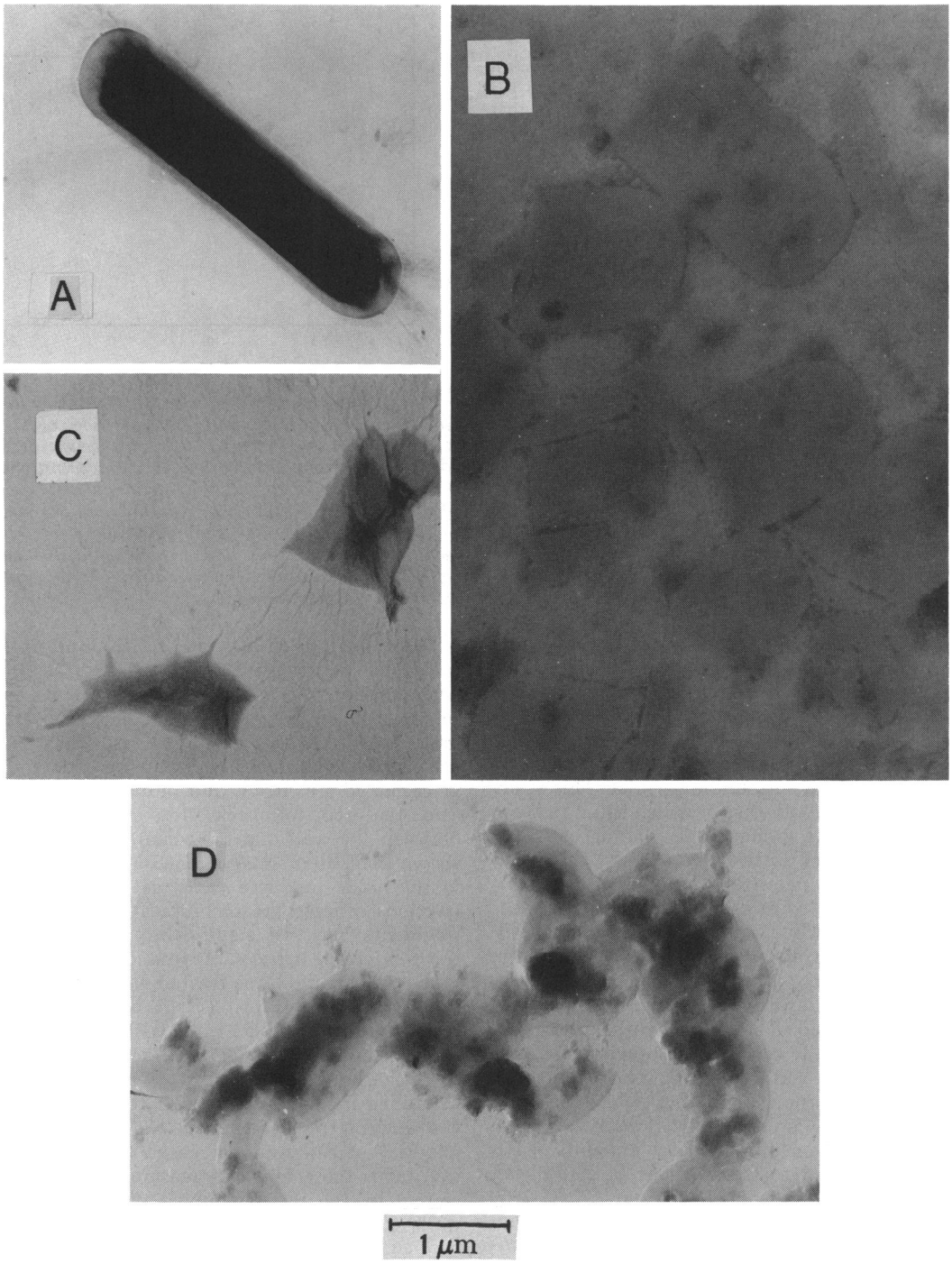

FIG. 1. *Electron micrographs of intact cell (A), crude wall (B), B_{12}-RL-wall (C), and RLH-wall (D) preparations.*

(2 by 78.5 cm). The B_{12} complex in the equilibration buffer (1.5 ml) was layered on the top and eluted. The elution profile was monitored with a Uvicord monitor (LKB-Produkter, Stockholm, Sweden) at 280 nm. The void and total volumes were determined by using blue dextran 2000 and B_{12}, respectively. Pepsin, soybean trypsin inhibitor, and cytochrome *c* were used as reference standards in molec-

ular weight determination of the B_{12} complex, as described by Andrews (1) and Whitaker (22).

Disc electrophoresis. Polyacrylamide gel electrophoresis of the B_{12} complexes was performed at *p*H 4.0 as described by Reisfeld et al. (15), except that the direction of electric current was reversed.

Absorption spectra of the B_{12} complexes. Absorption spectra of the purified B_{12} complexes in PBN were recorded by using a Beckman DK-2 spectrophotometer.

Chemicals. Chemicals were obtained from the following sources: vitamin B_{12} (cyanocobalamin), Roussel-Uclaf, Paris, France; ^{60}Co-labeled B_{12}, Radiochemical Centre, Amersham, England; blue dextran 2000, Pharmacia; cytochrome *c* (horse heart, type III), pepsin (two times crystallized), ribonuclease A (bovine pancreas, five times crystallized), trypsin (bovine pancreas, two times crystallized, type-1), and trypsin inhibitor (soybean, type 1-s), Sigma Chemical Co., St. Louis, Mo.; lipase MY (10,300 units/g), Meito Co., Tokyo; Pronase P, Kahen Kagaku Co., Tokyo; papain (two times crystallized, suspension), Worthington Biochemical Corp., Freehold, N.J. Hog intrinsic factor was generously supplied by L. Ellenbogen of Lederle Laboratories.

RESULTS

B_{12}-binding capacities of cells and walls of some lactobacilli in relation to their B_{12} requirements. A previous paper (13) has shown that the cells of B_{12}-requiring lactobacilli such as *L. delbrueckii* and *L. leichmannii* possess a specific activity to take up and preserve B_{12} from surrounding media. This study is extended to other strains belonging to these species. Figure 2 shows the binding of B_{12} to resting cells of *L. leichmannii* ATCC 7830 at increasing concentrations of B_{12}. The B_{12} bound to the cells was not released by washings with distilled water or buffers at *p*H values within physiological range. The maximum binding ability of this organism was 0.52 μg per mg of cells (dry weight) under the conditions described. The results with other organisms (Table 1) confirm the previous findings that the cells of B_{12}-requiring strains alone can bind large amounts of B_{12}. Unexpectedly, however, the isolated crude walls of these strains could not bind as large amounts of B_{12} as were bound to the walls of *L. delbrueckii* no. 1 (17, 18) and *L. leichmannii* ATCC 7830 (Table 1). The amounts of B_{12} estimated from radioactivity and by bioassay using *L. leichmannii* ATCC 7830 were entirely equal.

The cellular B_{12}-binding activity of *L. leichmannii* was destroyed partially by heating of cells for 10 min at 60 C (residual activity = 27%) and almost completely by heating at 100 C (residual activity = 3%).

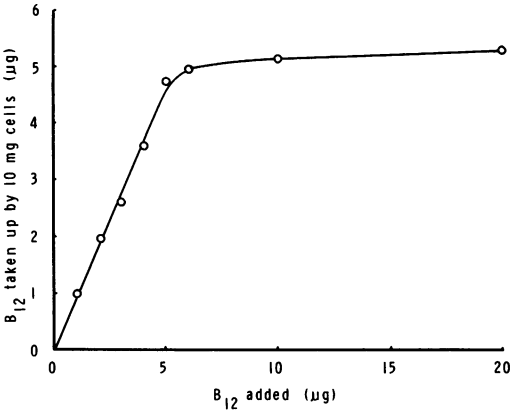

FIG. 2. *Amounts of B_{12} taken up by Lactobacillus leichmannii cells at increasing concentrations of B_{12}. A cell suspension (10 mg of cells, dry weight) was added to solutions with various B_{12} concentrations in total volumes of 10 ml. The reaction mixture was placed, with occasional stirring, in the dark at room temperature for 30 min. Then, the cells were collected by centrifugation and washed with distilled water. The radioactivity was measured, and was calculated as the quantity of B_{12}.*

Distribution of B_{12} in L. leichmannii cells. To investigate the cellular site for B_{12} accumulation, *L. leichmannii* cells grown in medium which had been supplemented with a large amount of B_{12} were disrupted, fractionated, and analyzed for B_{12} contents. Contents of carbohydrate, RNA, and hexosamines were also analyzed to check cross-contamination between the wall and ribosomal fractions. As shown in Table 2, the contents of hexosamines in the crude wall and particulate (ribosomal) fractions differed greatly, indicating that wall fragments may not significantly contaminate the particulate (ribosomal) fraction. When the crude wall fraction was treated with ribonuclease, lipase, and trypsin, RNA became undetectable in it; the facts exclude the possibility of the contamination of the fraction by ribosomes. A large part of the B_{12} which had been contained in the crude fraction remained after the enzymatic treatments (RLT-wall). The quantity of cell wall decreased to 18%, however, after the enzymatic treatments. This was caused by the rapid autolysis of the isolated walls of this organism in the buffer used for the enzymatic treatments. [This autolytic property differs greatly from *L. delbrueckii* no. 1 (20).] Thus, the decreased quantity does not necessarily indicate the removal of materials other than the wall, but rather represents removal of the wall itself.

Properties of B_{12} binder in L. leichmannii

TABLE 1. *B_{12}-binding capacities of cells and walls of lactobacilli*[a]

Strain	B_{12} requirement[b]	B_{12} bound to cells [μg/mg (dry wt)]	Yield of crude walls (% of cells)	B_{12} bound to crude walls [μg/mg (dry wt)]
Lactobacillus delbrueckii				
AHU 1056	No	0.019		
ATCC 9649	Yes	0.190	45.7	0.016
ATCC 11978	No	0.016		
ATCC 11979	No	0.051	23.7	0.008
Hyôgo-jôzô	Yes	0.150	22.9	0.036
IAM 1085	No	0.013		
IAM 1149	Not determined	0.260	50.3	0.440
IAM 1174[c]	Yes	0.220	30.4	0.050
IAM 1197[c]	Yes	0.240	41.0	0.196
IAM 1928	No	0.019		
IFO 3534	Yes	0.155	26.8	0.100
L. casei subsp. rhamnosus				
ATCC 11443[d]	No	0.020		
L. leichmannii				
ATCC 7830	Yes	0.520	21.2–27.4	0.880

[a] The organisms were grown at their optimal temperatures in appropriate media, harvested, washed, and fractionated as *L. leichmannii*. B_{12}-binding capacities of cells and isolated walls were measured as in Fig. 2.

[b] B_{12} requirement was determined by using B_{12} assay medium USP.

[c] IAM 1174 formerly referred to as *L. delbrueckii* no. 2; IAM 1197 formerly *L. delbrueckii* no. 3.

[d] Formerly *L. delbrueckii* ATCC 11443.

TABLE 2. *Distribution of B_{12} within the cell*[a]

Cellular component	Dry wt (g)	Total sugars (mg)	Ribonucleic acid (mg)	Hexosamines (mg)	B_{12} (μg)
Whole cell	1.24 (100)[b]	31.8	319.8	13.7	292.9
Crude walls	0.44 (35.5)	16.8	81.8	11.6	214.8
Particulate	0.25 (20.2)	8.4	108.0	0.3	70.6
Soluble part	0.55 (44.3)	6.6	130.0	1.8	7.5
RLT-walls	0.08 (6.5)		0		131.0

[a] Cells grown in 2 liters of inoculation medium for *L. leichmannii* containing 1 mg of B_{12} were harvested at the early stationary phase of growth, washed with 10 mM Tris-succinate buffer (*p*H 7.2) containing 5 mM magnesium acetate, and fractionated as described in Materials and Methods after repeated sonic treatments (18). A part of the crude wall fraction received enzymatic treatments (RLT-walls).

[b] Values in parentheses are percentages.

walls. Properties of the B_{12} binder in the wall of *L. leichmannii* were investigated by using several hydrolytic enzymes or HCl solutions, or both. Table 3 shows that B_{12}, once bound to the wall, was not significantly liberated by treatments with the enzymes. Treatments with 0.2 N HCl resulted in the disappearance of B_{12} from the wall in the presence or absence of pepsin. Supernatant solutions which were derived from these 0.2 N HCl treatments contained nondialyzable B_{12}, suggesting that the B_{12} was in bound form with the binder liberated from the wall. When the wall was treated with enzymes such as pepsin, Pronase, or trypsin before the binding of B_{12}, the ability of the wall to bind B_{12} was almost completely lost (Table 4). The same was true for the wall treated with 0.2 N HCl in the presence or absence of pepsin. However, in the case of 0.2 N HCl treatment without pepsin, the supernatant solution contained a nondialyzable B_{12} binder. The difference in results given in Tables 3 and 4 may be interpreted as follows. B_{12} is bound to the same, or very close, position in the wall as that on which enzymes such as pepsin, Pronase, or trypsin act; thus, once bound, B_{12} could not be liberated by treatments with these enzymes because of steric hindrance by

TABLE 3. *Liberation of B_{12} from the wall by various treatments*[a]

Treatment	Dry wt after treatment (mg)	B_{12} in treated walls (μg)	Nondialyzable B_{12} in supernatant solution (μg)
0.02 N HCl, pepsin	14.9	36.6	
0.2 N HCl, pepsin	15.2	1.0	20.0
0.02 N HCl, none	36.9	37.2	
0.2 N HCl, none	28.8	2.4	31.4
P buffer, papain	19.0	41.7	
P buffer, Pronase	16.6	23.8	3.8
P buffer, trypsin	16.4	25.4	3.7
P buffer, lipase	15.3	41.2	
P buffer, ribonuclease	15.4	40.6	
P buffer, none	16.0	41.7	
Distilled water, 0 C, none	46.8	40.1	

[a] Crude walls to which B_{12} had been fully bound were washed with distilled water and resuspended in HCl, 0.05 M phosphate buffer (P buffer), *p*H 7.0, or distilled water. Where indicated, enzymes (2 mg each, except 1 mg of ribonuclease) were added in final volumes of 7.5 ml. Cysteine hydrochloride (4 mg) was added in the papain reaction mixture. The reaction mixtures were incubated at 37 C for 48 hr in the dark, except that the tube containing the distilled water suspension was placed at 0 C for the same period. Drops of toluene were added as a preservative. The treated walls were collected by centrifugation and washed with distilled water, and radioactivity was measured. Dry weights were also determined. The supernatant solutions were dialyzed against distilled water at 4 C for 72 hr, and radioactivity remaining in the cellophane bags was measured. Free B_{12} was dialyzed as a reference.

TABLE 4. *B_{12}-binding abilities of walls treated with various agents*[a]

Treatment	Dry wt after treatment (mg)	B_{12} bound to treated walls (μg)	B_{12} bound to supernatant solution (μg)
0.02 N HCl, pepsin	11.4	1.0	1.2
0.2 N HCl, pepsin	15.5	1.0	1.9
0.02 N HCl, none	34.5	21.5	
0.2 N HCl, none	30.1	0.9	12.8
P buffer, papain	18.3	21.0	
P buffer, Pronase	15.0	0.4	1.8
P buffer, trypsin	15.7	0.6	1.5
P buffer, lipase	13.4	19.4	
P buffer, ribonuclease	15.7	17.3	
P buffer, none	13.4	21.0	
Distilled water, 0 C, none	40.3	28.0	

[a] Crude walls were first treated with enzymes as described in the footnote to Table 3. They were collected by centrifugation, washed with distilled water after the above treatments, and then treated with 60 μg of B_{12} in a total volume of 7.5 ml with 0.05 M phosphate buffer (P buffer), *p*H 7.0. Radioactivity was measured for B_{12} bound to the walls, as in Fig. 2. The supernatant solutions of enzymatic or HCl treatments were put in cellophane bags and dialyzed against distilled water at 4 C for 16 hr until they reached neutral *p*H. Then, 60 μg of B_{12} was added to each, with subsequent incubation for 1 hr at room temperature and dialyzation against distilled water at 4 C for 72 hr. The remaining radioactivity was measured.

the B_{12}. An alternative possibility is the conformational change of a polypeptide, which contains a B_{12}-binding portion in the wall, upon binding with B_{12}. The change prevents the proteolytic actions on the B_{12}-binding portion which is usually sensitive to these enzymes. The enzyme could digest the B_{12}-binding portion before the binding of B_{12}. Treatment with 0.2 N HCl could liberate a B_{12} complex from the wall retaining B_{12}, or a B_{12} binder from the wall retaining no B_{12}. Similar phenomena have been observed with *L. delbrueckii* (19). Lipase and ribonuclease had no significant effect on the B_{12}-binding activity of the wall.

The wall which was incubated only in phosphate buffer showed a remarkable loss of materials sedimentable by centrifugation, indicating that autolysis took place. However, the amount of B_{12} which had been bound to the wall was not reduced by this decrease in the quantity of the wall (Table 3); neither was the B_{12}-binding ability significantly affected by the decrease (Table 4). The facts suggest that the part of the wall that is susceptible to autolysis has no relation to the B_{12}-binding site.

Physicochemical properties of the B_{12} complex isolated from the wall. An elution pattern of the B_{12} complex on CM-cellulose column chromatography is shown in Fig. 3. Although the B_{12} complex was eluted as a single peak, the ratio of optical densities at 361 nm (absorption owing to B_{12} moiety) and 280 nm (polypeptide moiety) differed slightly at the top and at both extremities of the peak, suggesting that the B_{12} complex was microheterogeneous. The B_{12} complex could be purified by rechromatography, which was subsequently concentrated by using a small column of CM-cellulose and dialysis. This showed a single band on polyacrylamide gel electrophoresis (Fig. 4). The final yield of the B_{12} complex was 1.7 to 2.7 mg/g of cells (dry weight). The B_{12} complex was insoluble in salt-free water and soluble in an NaCl solution above 0.05 M concentration. The B_{12} complex contained neither

carbohydrate nor RNA, as revealed by the anthrone (24) and orcinol (3) methods, by subtracting values attributable to the B_{12} moiety or by measuring directly those values in the B_{12} binder. The B_{12} binder was obtained by separation of the B_{12} complex into B_{12} and the binder by using the procedures of Gräsbeck et al. (5). The B_{12} complex consisted of B_{12} and polypeptide. On Sephadex G-75 column chromatography, the B_{12} complex was eluted just where soybean trypsin inhibitor was eluted (Fig. 5). Since the molecular weight of soybean trypsin inhibitor is 21,500 (23), that of the B_{12} complex should be around this value. Minimum molecular weight of the B_{12} complex, estimated by measuring the content of B_{12} from an optical density at 361 nm and the content of protein by the method of Lowry et al. (14), agreed well with this value. Microheterogeneity in the ratio of optical densities at 280 and 361 nm was also observed in this chromatographic elution pattern.

Resemblance of the absorption spectra (Fig. 6) of the B_{12} complex to those of B_{12} (cyanocobalamin) over the range from 330 to 600 nm clearly indicates that the complex contained B_{12}. Slight shift of peaks around 361 and 550 nm toward shorter wavelengths was observed for the B_{12} complex. The increased peak at 278 nm of the B_{12} complex indicates involvement of polypeptide in this complex. Vitamin B_{12} showed a shift of the peak at 361 to 368 nm when KCN was added at a final concentration of 0.1 M (*p*H 11.0), whereas the B_{12} complex (peak at 360 nm in this case) did not. This fact suggests that the position for entry of the sec-

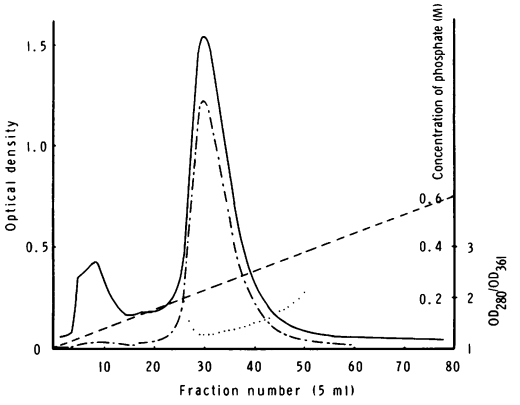

FIG. 3. *CM-cellulose column chromatography of the B_{12} complex of the wall. A B_{12} complex solution in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl (PBN; 145 ml with optical densities: 280 nm = 1.346, 361 nm = 0.496) was placed on a CM-cellulose column (1.2 by 13 cm) equilibrated with PBN. The elution was performed as described in Materials and Methods. Symbols: optical density at 280 nm (—) and at 361 nm (- - -); ratio of optical densities at 361 and 280 nm (· · ·); concentration of phosphate buffer (- - -).*

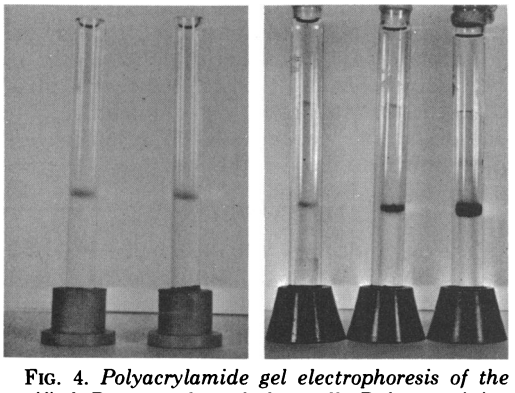

FIG. 4. *Polyacrylamide gel electrophoresis of the purified B_{12} complex of the wall. Before staining (left), the color of the band was red. After staining with amido black (right), the color of the band was blue-black.*

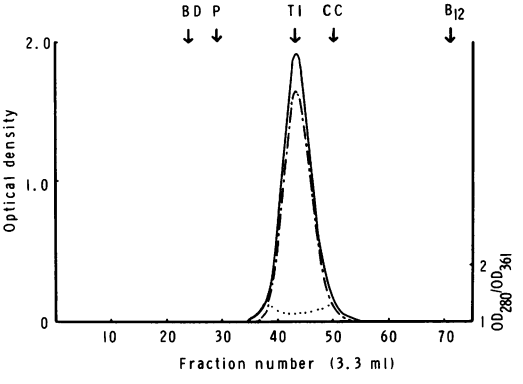

FIG. 5. *Gel filtration on Sephadex G-75 of the purified B_{12} complex of the wall. Symbols: optical density at 280 nm (—) and at 361 nm (- - -); ratio of optical densities at 361 and 280 nm (· · ·). Arrows indicate where blue dextran (BD), pepsin (P), trypsin inhibitor (TI), cytochrome c (CC), and free B_{12} (B_{12}) were eluted as peaks.*

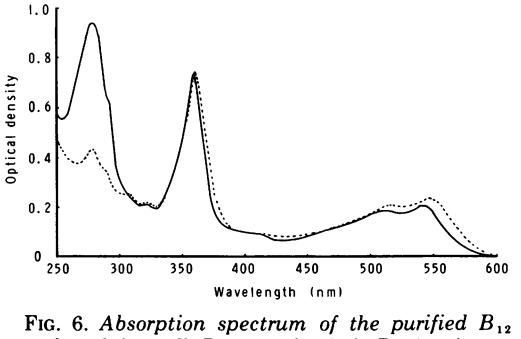

FIG. 6. *Absorption spectrum of the purified B_{12} complex of the wall. B_{12} complex (—), B_{12} (- - -).*

ondary cyano group in the B_{12} moiety was not available. This may have been a result of the involvement of this position for binding directly to the polypeptide moiety, or of some conformational changes of the B_{12} moiety, caused upon binding to the polypeptide, which hindered the entrance of more cyano group to form dicyanocobalamin with the B_{12} molecule. The same phenomenon has been observed with the B_{12} complex of *L. delbrueckii* (20). When the *p*H of the B_{12} complex solution was brought to *p*H 12.9 with Gräsbeck's buffer consisting of NaOH, glycine, and NaCl (5), the peak at 360 nm shifted to 368 nm upon addition of KCN, indicating that the B_{12} moiety received the secondary cyano group. The addition of Gräsbeck's buffer alone did not cause any shift of the peak at 360 nm of the B_{12} complex or at 361 nm of B_{12}. Neutralization of the alkaline KCN solution (*p*H 12.9) of the B_{12} complex caused a shift of the peak at 368 nm back to 360 nm. Therefore, the B_{12} complex should be dissociated into free B_{12} and polypeptide at *p*H 12.9 to be ready to receive a cyano group, as was revealed more clearly by using the procedures of Gräsbeck et al. (5). This method consisted of eluting the B_{12} complex through an alkali-equilibrated Bio-Gel P-10 column. The detached polypeptide and B_{12} eluted separately from the column. The free polypeptide thus obtained could bind B_{12} again at neutral *p*H.

Effect of B_{12} binders on B_{12} binding to L. leichmannii and other bacterial species. The B_{12} binder, isolated from *L. leichmannii* walls, or hog intrinsic factor was added together with B_{12} to the cell suspensions of *L. leichmannii* or other bacterial species having no B_{12}-binding activity. *L. leichmannii* cells took up B_{12} in the presence of the B_{12} binder as well as in its absence. On the other hand, hog intrinsic factor, used as a reference, interfered with the B_{12} binding to the cells (Table 5). *Escherichia coli* cells took up large amounts of B_{12} in the presence of the B_{12} binder isolated from *L. leichmannii*, suggesting that B_{12} was bound to *E. coli* cells through intermediation of the B_{12} binder. The amount of B_{12} taken up by cells such as *L. delbrueckii* and *Streptococcus faecalis* with the addition of B_{12} binder was no greater than the amount taken up by these cells with no addition of B_{12} binder.

Properties of the B_{12} complex isolated from ribosomes. A B_{12} complex quite similar to that of the wall was obtained from the *L. leichmannii* particulate (ribosomes) by treatment with 0.2 N HCl using the same procedures employed for isolation of the B_{12} complex from the wall.

TABLE 5. *Effect of B_{12} binders on binding of B_{12} to bacterial cells*[a]

Organism	B_{12} bound to cells in the presence of		
	No binder (μg)	B_{12}-binder[b] (μg)	Hog IF[c] (μg)
Escherichia coli no. 215[d]	0.15	4.35	
Lactobacillus delbrueckii AHU 1056	0.17	0.08	
L. leichmannii ATCC 7830	3.52	2.88	0.015
Streptococcus faecalis ATCC 8043	0.03	0.08	

[a] The organisms were grown at their optimal temperatures in appropriate media, harvested, and washed as *L. leichmannii*. A washed-cell suspension (10 mg dry weight) was added to a reaction mixture containing 5 μg of B_{12}, 1 mmole of phosphate buffer (*p*H 7.0), and the B_{12}-binder derived from *L. leichmannii* walls or hog intrinsic factor, in a final volume of 10 ml. The reaction mixture had been allowed to stand for 10 min before addition of the cell suspension. After 30 min of incubation, the cells were collected by centrifugation and washed with the same buffer, and radioactivity was measured as in Fig. 2.

[b] Obtained from *L. leichmannii* walls retaining no B_{12} by treatment with 0.2 N HCl and used in an amount sufficient to bind 5.2 μg of B_{12}.

[c] Hog intrinsic factor in an amount sufficient to bind 5.2 μg of B_{12}.

[d] A B_{12}-requiring mutant (6).

The B_{12} complex from ribosomes was eluted as single peaks on CM-cellulose or Sephadex G-75 column chromatography, or both, but it was microheterogeneous, as judged from the ratio of optical densities at 280 and 361 nm, at the top and at both extremities of the peak as was B_{12} complex from the wall. However, it moved as a single band on polyacrylamide gel electrophoresis. The absorption spectrum of the B_{12} complex and the molecular weight (ca. 21,500) estimated from the chromatographic elution on the Sephadex G-75 column were quite similar to those of the wall.

DISCUSSION

Kashket and Beck (7) questioned whether the B_{12}-binding ability of our previous cell wall preparation of *L. delbrueckii* no. 1 (17, 18) may have been attributable to a B_{12}-binding protein detached from ribosomes and co-precipitated with the wall preparation during cell fractionation. They stated that we did not show that our wall preparation was free from contamination by ribosomal materials. Their statements are not exact. We washed the wall

preparation several times by centrifugation under conditions in which ribosomes were not precipitated and successively treated the preparation with trypsin, ribonuclease, and lipase (17, 18, 20). These treatments may have cleaved off materials other than the wall. Electron micrographs also showed that our wall preparation was free from electron-dense materials, namely, cytoplasmic components. Moreover, approximately two-thirds of the B_{12} bound to the cell was released into the surrounding medium when it was transformed to the protoplast. This fact provided additional evidence that the wall possesses B_{12}-binding ability.

Kashket and Beck (7) did not isolate the cell wall fraction nor did they examine the B_{12} binding ability of their strain, *L. leichmannii* ATCC 7830. The results of the present investigation clearly indicate that the wall is a "primary reservoir" of B_{12} (17, 18) in this organism. Three-fourths of the B_{12} bound to the cell was found in the crude wall fraction. This amount of B_{12} was not decreased significantly by treating the fraction with ribonuclease, lipase, and trypsin (Table 2). The possibility of contamination of the wall fraction by ribosomes or co-precipitated ribosomal B_{12} binders is safely excluded by measuring the contents of B_{12}, RNA, and hexosamines in the wall and ribosomal (particulate) fractions. The fact that the ratio of B_{12} contents in the wall and particulate fractions was 3:1 and that of hexosamines was 39:1, together with the finding that the enzymatically "purified wall" fraction (RLT-wall) retained three-fifths of the initially bound B_{12} and no RNA, emphasizes the B_{12}-binding capacities of the wall and of the ribosomes. The ribosomes (particulate) were responsible for approximately one-fourth of the B_{12} bound to the cell. It appears quite unlikely that most of the ribosomal B_{12} binders were released during cell fractionation procedures and bound to the wall fraction. In fact, the B_{12} bound to ribosomes could not be released by washings under the conditions routinely employed. The wall fraction obtained from *L. leichmannii* cells retaining no B_{12} also possessed the B_{12}-binding capacity (Table 1).

B_{12} was shown to bind, in the wall, to a polypeptide on which pepsin, Pronase, and trypsin act (Tables 3 and 4).

The previous findings that B_{12}-requiring lactobacilli have sufficient specific activity to take up large amounts of B_{12} were also confirmed in the present study (Table 1). However, it was found that the cellular capacities for B_{12} binding cannot be accounted for by the B_{12}-binding activity of the walls alone in these lactobacilli strains; some other cellular site, probably ribosomes, may exist as a principal site for B_{12} accumulation. This situation may be interpreted as an evolutional cellular differentiation of these bacterial strains into walls and ribosomes with respect to the B_{12}-accumulating sites. The fact that quite similar B_{12} binders were obtained from the wall and ribosomes of *L. leichmannii* may support this interpretation.

A B_{12}-polypeptide complex (the B_{12} binder to which is bound B_{12}) was isolated from the wall and purified. Its molecular weight was estimated to be around 21,500. A B_{12}-polypeptide complex obtained from the ribosomes was quite similar to that obtained from the wall with respect to absorption spectrum, electrophoretic mobility, and molecular weight (ca. 21,500).

The B_{12} binder that was isolated from the wall of *L. leichmannii* retaining no B_{12} did not significantly affect binding of B_{12} to the cell of the same organism when added to the reaction mixture for B_{12} binding (Table 5). The possibility that ribosomal B_{12} binders were bound to the wall preparation, thus giving an apparent B_{12}-binding ability to the preparation, can safely be excluded if we examine carefully the values obtained. The amount of B_{12} bound to the cell was 3.52 μg at *p*H 7.0 (0.1 M phosphate buffer) in the absence of B_{12} binder (control), whereas this amount was 2.88 μg in the presence of the B_{12} binder. If ribosomal B_{12} binders were responsible, the latter value should exceed the control value of 3.52 μg. It should be remembered that cellular fractionation was carried out at a *p*H at which B_{12} binding was not increased if "released B_{12}-binders" were present. Hog intrinsic factor completely inhibited B_{12} binding to *L. leichmannii* cells, as described by Gräsbeck (4). In contrast, the fact that the B_{12} binder derived from the wall did not significantly reduce the B_{12} binding to *L. leichmannii* cells is compatible with previous findings that a B_{12}-polypeptide complex derived from *L. delbrueckii* no. 1 wall can be utilized by *L. delbrueckii* for growth (17, 20).

In view of the present findings, the wall of *L. leichmannii* ATCC 7830 plays a role as a "primary reservoir" of B_{12} as proposed earlier with *L. delbrueckii* (17, 18), because B_{12}, once bound to the wall, should enter the cytoplasm to exhibit its biological functions. The mechanism by which B_{12}, once bound to the wall, is transported to the ribosomes (17) awaits further studies.

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