EFFECTS OF DIVALENT CATIONS ON VITAMIN B₁₂ ADSORPTION TO BRUSH BORDERS OF RAT INTESTINE¹

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Summary A brush border preparation from rat intestine was incubated with rat intrinsic factor-vitamin B₁₂ complex in 0.01 M Tris-HCl buffer, pH 7.4. The ⁵⁷Co-B₁₂ uptake to brush borders was proportional to the amount of protein or to alkaline phosphatase activity in the preparations. The uptake increased with time of incubation. At 37°C, the uptake after incubation for 15 min was 80–85% of that for one hr. The uptake at 4°C was approximately 70% of that at 37°C. There was no difference as a result of adding glucose to the incubation medium. The uptake was observed in the alkaline environment above pH 6.3. Maximum uptake occurred at pH 8.0. Brush borders washed with Krebs-Ringer bicarbonate buffer (pH 7.4) exhibited no difference in B₁₂ uptake, whether in the presence or absence of calcium ion. But brush borders washed with ethylenediaminetetraacetate exhibited no uptake when incubated in calcium-free medium. The uptake reached a maximum by addition of calcium ion at a concentration of 0.3 mM, and was not alter up to 10 mM. Addition of magnesium ion exhibited no uptake. Calcium-dependent B₁₂ uptake was markedly inhibited by manganese ion. Magnesium ion seemed to slightly inhibit the calcium-dependent uptake.

It is well known that pernicious anemia occurs with deficiency of vitamin B₁₂. Foods contain very small amounts of vitamin B₁₂ and the daily requirement of the vitamin is much lower than those of other vitamins (about 3 µg per day). In order to absorb such a small amount of vitamin B₁₂, a specific absorptive mechanism exists. Apart from other vitamins, the physiological absorption of vitamin B₁₂ is mediated by an intrinsic factor (IF), a mucoprotein secreted from parietal cells of the stomach. There are a number of reviews on B₁₂ absorption (1–7). Vitamin B₁₂ combines with IF to form an IF-B₁₂ complex which attaches itself

¹ Studies on Vitamin B₁₂ Absorption.

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to the brush border of the small intestine. It has been generally postulated that there might be a specific receptor in the microvillous membranes of the brush border (8-10), but the properties of such a receptor have not yet been clarified.

Rats have been employed as models for IF-mediated B_{12} absorption in man because physiological absorption of vitamin B_{12} in rats is also mediated by IF (11, 12). Vitamin B_{12} is absorbed from the distal portion of the ileum in man, but from the middle portion of the small intestine in rats (13-16). From the results of *in vitro* experiments with everted sacs, intestinal loops and mucosal homogenates, it has been found that IF-mediated B_{12} absorption requires calcium ion and is inhibited by ethylenediaminetetraacetate (EDTA) (10, 16-20). There are some reports that magnesium ion can be partially substituted for calcium ion, or that the inhibited absorption by EDTA is recovered after magnesium administration (20, 21).

The B_{12} uptake to everted sacs or mucosal homogenates from rat, guinea pig or human small intestine occurs at an alkaline pH (20, 22, 23). The B_{12} uptake to everted sacs decreased in anaerobic conditions, in glucose-free medium or at low temperatures (24), but the uptake to mucosal homogenates does not require the presence of oxygen or glucose (22).

DONALDSON *et al.* (25) demonstrated that the adsorption of IF- B_{12} to brush borders or microvillous membranes of hamster small intestine was not diminished by the removal of glucose or oxygen from the incubation medium, and that it was not significantly altered when the temperature of incubation was lowered from 37 to 7°C. They also observed that the adsorption was markedly reduced by the removal of calcium and magnesium ion from the incubation medium.

In this paper the effect of divalent cations on B_{12} adsorption to brush borders of rat intestine was studied more precisely. In order to observe the effect of each cation, 0.01 M Tris-HCl buffer was used as the incubation medium. The effects of the pH of the medium, incubation time and temperature, and glucose in the medium were also studied.

MATERIALS AND METHODS

1. *Preparation of intestinal brush borders of rat (Table 1).* The brush border was prepared by the method of MILLER and CRANE (26) with a modification. Wistar rats weighing 250-350 g were sacrificed after fasting 48 hr. The entire small intestine was cut into several pieces and everted over a glass bar. After washing with cold saline, the mucosal surface was scraped with a glass slide. Scrapings of intestines from two rats was added to 100 ml of cold 5 mM EDTA-NaOH buffer (pH 7.4). It was homogenized in a Waring blender at a low speed for 25 sec. The homogenate was filtered three times through two layers of surgical gauze to eliminate mucus. The filtrate was then centrifuged at $500 \times g$ (2,100 rpm) for 10 min in a refrigerated centrifuge (Tominaga No. 90 UV). The sediment was

Table 1. Preparation procedure for the brush border of rat.

suspended in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, washed twice and centrifuged in the same manner. The sediment was re-suspended in cold KRB and centrifuged at low speed (500 rpm) for one min. The sediment was discarded and the supernatant was filtered through two layers of surgical gauze to eliminate the aggregated materials. The filtrate was used as the brush border suspension for the following experiments.

The amount of protein was determined by the method of LOWRY *et al.* (27) after the material was solubilized in 0.5 N NaOH. Alkaline phosphatase activity was measured by the method of BESSEY *et al.* (28) with a modification. Paranitrophenylphosphate was used as the substrate and the reaction was stopped after 15 min incubation at 37°C.

For electron microscopic examination, the sediment obtained by centrifuging the brush border suspension was fixed in 4% glutal aldehyde, refixed in 2% OsO₄, dehydrated with alcohol and propylene oxide and embedded in Epon 812 in the usually manner.

2. *Preparation of rat intrinsic factor solution.* The gastric mucosal scrapings from two fasted rats were added to 10 ml of cold saline and homogenized in

a Waring blender. The homogenate was centrifuged at $8,000 \times g$ for 15 min. The pH of supernatant solution was raised to 10.0 by addition of 0.05 N NaOH solution. After standing at 4°C for one hr, it was neutralized to pH 7.4 with 0.05 N HCl solution and then centrifuged at $100,000 \times g$ for one hr in an ultracentrifuge (Hitachi 65P). The supernatant solution was stored at -20°C as the rat intrinsic factor solution.

The B_{12} binding capacity of the solution was measured by a method previously reported by us (29). The IF solution was diluted 10 times with saline and 0.05, 0.1, 0.2 and 0.3 ml aliquots of the diluted solution were put into test tubes in duplicate. Then, 0.6 ng of $^{57}\text{Co-B}_{12}$ and 5 ml of zirconium phosphate gel (pH 5.0) was added to each tube and mixed completely. Each tube was centrifuged at 2,000 rpm for 10 min, and the precipitate was washed four times with ammonium acetate buffer (pH 5.0). The radioactivity of each final precipitate was measured in a well-type scintillation counter. As the precipitate contains IF-bound B_{12} adsorbed to the gel, B_{12} binding capacity of IF solution was calculated from the radioactivity of the precipitate.

3. *Preparation of $^{57}\text{Co-B}_{12}$ solution and IF- B_{12} complex.* Radioactive cyanocobalamin ($^{57}\text{Co-B}_{12}$) was obtained from Radiochemical Center, Amersham, England. The specific activity was 166–200 $\mu\text{Ci}/\mu\text{g}$. It was diluted with non-radioactive cyanocobalamin to obtain a $^{57}\text{Co-B}_{12}$ solution containing 2 ng and about 100,000 cpm in each 0.4 ml. IF- B_{12} complex was prepared by mixing 0.4 ml of $^{57}\text{Co-B}_{12}$ solution with IF solution which was diluted with saline to obtain a binding capacity of 2 ng.

4. *Adsorption of vitamin B_{12} to brush borders.* Two different brush border preparations were used for the incubation experiments (Table 1). One was prepared by centrifuging the brush border suspension in KRB at 3,000 rpm for 10 min (A: non-treated brush border). The other was prepared by suspending the brush border (A) in 5 mM EDTA-NaOH buffer, pH 7.4, and further centrifugation (B: EDTA-treated brush border).

Brush border preparation (A) or (B) was suspended in 6 ml of incubation buffer and IF- B_{12} complex or B_{12} solution was added. After incubation, 5 ml of the same buffer was added and the preparation was centrifuged at $8,000 \times g$ for 10 min. The sediment was washed twice. The radioactivity of the final sediment was measured in a well-type scintillation counter. The pg of B_{12} taken up was calculated from the radioactivity of added $^{57}\text{Co-B}_{12}$ solution.

To study the relationship between B_{12} uptake and the amount of the brush border, brush borders (A) from 2.5, 5.0, 10.0 and 15.0 ml of suspensions were incubated at 37°C for one hr in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl and 10 mM CaCl_2 . All other experiments were performed using brush borders (A) or (B) from 10 ml of suspension. The effect of incubation time was studied at 37 and 4°C for 15, 30, 60 and 120 min. The effect of glucose was studied at 4, 20, 37 or 45°C in a medium containing glucose at a concentration of

2.5 mg/ml or in a medium containing no glucose. To study the effect of pH, incubation was performed in 0.01 M Tris-HCl buffer which had previously adjusted to various pHs.

Effects of cations were studied by incubating brush borders (A) or (B) in various buffers as follows: (a) KRB, (b) 0.01 M Tris-HCl buffer containing 0.9% NaCl, (c) 0.01 M Tris-HCl buffer containing 0.9% NaCl and 10 mM CaCl₂, (d) 5 mM EDTA-NaOH buffer, (e) 5 mM EDTA-NaOH buffer containing 15 mM CaCl₂, (f) 0.01 M Tris-HCl buffer containing 0.9% NaCl and 10 mM KCl, (g) 0.01 M Tris-HCl buffer containing 0.9% NaCl and 10 mM MgCl₂, (h) 0.01 M Tris-HCl buffer containing 0.9% NaCl and 10 mM MgSO₄, (i) 0.01 M Tris-HCl buffer containing 0.9% NaCl and 10 mM MnCl₂, (j) 0.01 M Tris-HCl buffers containing 0.9% NaCl and various concentrations (0.1, 0.2, 0.3, 0.5, 1.0, 5.0 and 10.0 mM) of CaCl₂, (k) 0.01 M Tris-HCl buffer containing 0.9% NaCl, 10 mM CaCl₂ and 20 mM MgCl₂, (l) 0.01 M Tris-HCl buffer containing 0.9% NaCl, 10 mM CaCl₂ and 20 mM MnCl₂, (m) 0.01 M Tris-HCl buffers containing 0.9% NaCl, 2.5 mM CaCl₂ and various concentrations (1, 5, 10 and 20 mM) of MgCl₂, (n) 0.01 M Tris-HCl buffers containing 0.9% NaCl, 2.5 mM CaCl₂ and various concentrations (1, 5, 10 and 20 mM) of MnCl₂, (o) 0.01 M Tris-HCl buffers containing 0.9% NaCl and various concentrations (1, 5, 10 and 20 mM) of MgCl₂, (p) 0.01 M Tris-HCl buffers containing 0.9% NaCl and various concentrations (1, 5, 10 and 20 mM) of MnCl₂. All buffers were adjusted to pH 7.4.

RESULTS

1. *Alkaline phosphatase activity of the brush border preparation*

Brush border preparations used for the experiments contained 0.75 ± 0.29 mg of protein and had 2.80 ± 1.11 μ M of alkaline phosphatase activity. The relationship between amount of protein and alkaline phosphatase activity exhibited a significantly positive correlation. The correlation coefficient was 0.721, and the specific activity of alkaline phosphatase was 3.87 ± 1.39 μ M/mg protein (Fig. 1).

Electron microscopic findings of the prepared brush borders showed them to be almost pure though there was some contamination by cell debris and other organelles. Sheets of microvilli were preserved to the depth of the terminal web of the absorptive cell. The structural integrity of the individual microvilli was maintained well also after incubation (Fig. 2).

2. *Relationship between B₁₂ uptake and amount of brush borders*

The B₁₂ uptake was proportional to the amount of protein or to alkaline phosphatase activity of incubated brush borders in the incubation with IF-B₁₂ complex. However, the uptake was scarcely seen in the incubation with B₁₂ alone (Fig. 3). The pg uptake of B₁₂ per mg protein or per μ M alkaline phosphatase are shown in the following experiments.

Fig. 1. Relationship between alkaline phosphatase activity and amount of protein in each 10 ml of the brush border suspension.

Fig. 2. Electron micrograph of the brush borders (A) before and (B) after incubation at 37°C for 1 hr ($\times 7,500$).

Fig. 3. Relationship between ⁵⁷Co-B₁₂ uptake and alkaline phosphatase activity of the incubated brush border. The sediment of 2.5, 5.0, 10.0 or 15.0 ml of the brush border suspension was incubated with IF-B₁₂ complex or B₁₂ alone at 37°C for 1 hr.

Fig. 4. Effects of the incubation time and temperature on ⁵⁷Co-B₁₂ uptake to brush borders. Brush borders were incubated with IF-B₁₂ in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.9% NaCl and 10 mM CaCl₂ at 4 or 37°C for 15, 30, 60 or 120 min.

3. Effects of incubation time and temperature, and glucose on the incubation medium

The B₁₂ uptake increased gradually with time of incubation at 37 or 4°C. As early as 15 min, the uptake at 37°C already reached the level of 80–85% of the one incubated for one hr, while the uptake at 4°C was 50–55% (Fig. 4).

As for the effect of temperature, the uptake was maximal at 20°C, and at 37°C it was about 90% of that at 20°C. The uptake at 4°C was similar to that at 45°C, and about 70% of that at 37°C. No difference could be found between the presence or the absence of glucose at each temperature studied (Fig. 5).

4. Effect of pH on the incubation medium

Though B₁₂ uptake did not occur at under pH 6.0, it began to occur at pH 6.3

Fig. 5. Effect of glucose in the incubation medium on $^{57}\text{Co-B}_{12}$ uptake. Brush borders were incubated with IF- B_{12} for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing 2.5 mg/ml of glucose or containing no glucose at 4, 20, 37 or 45°C. Each data represents mean $\pm$ S.D.

Fig. 6. $^{57}\text{Co-B}_{12}$ uptake to brush borders at various pHs. Brush borders were incubated with IF- B_{12} at 37°C for 1 hr in 0.01 M Tris-HCl buffer previously adjusted to various pHs.

and reached a maximum at pH 8.0. At pH 9.0, the uptake was a little lower than that at pH 8.0 and was almost similar to that at pH 7.4 (Fig. 6).

5. Effects of various cations on B_{12} uptake to brush borders

The IF-mediated B_{12} uptake to brush borders (A) exhibited no difference between incubation in the Tris-HCl buffer and in KRB. Furthermore, the uptake occurred regardless of calcium ion in the incubation medium. However, the uptake did not occur when incubated in EDTA buffer, and it was restored by addition of excessive calcium ion to the same buffer (Fig. 7).

The uptake in brush borders (B) occurred when incubated in a medium con-

Fig. 7. ⁵⁷Co-B₁₂ uptake to brush borders incubated with IF-B₁₂ complex or B₁₂ alone at 37°C for 1 hr in the medium of (A) KRB, (B) 0.01 M Tris-HCl buffer, (B') 0.01 M Tris-HCl buffer containing 10 mM CaCl₂, (C) 5 mM EDTA-NaOH buffer, (C') 5 mM EDTA-NaOH buffer containing 15 mM CaCl₂. The pH of each buffer was 7.4.

Fig. 8. ⁵⁷Co-B₁₂ uptake to EDTA-treated brush borders incubated with IF-B₁₂ or B₁₂ alone at 37°C for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing (A) 0.9% NaCl, (B) 10 mM CaCl₂, (C) 10 mM KCl, (D) 10 mM MgCl₂, (D') 10 mM MgSO₄, or (E) 10 mM MnCl₂.

taining calcium ion, but did not occur in a calcium-free medium or in a medium containing potassium, magnesium or manganese ion instead of calcium ion (Fig. 8).

6. Effect of calcium concentration on B₁₂ uptake to brush borders

When brush borders (B) were incubated in the medium containing various concentrations of calcium ion, B₁₂ uptake increased linearly until 0.3 mM of CaCl₂ concentration. The uptake nearly reached maximum at 0.3 mM and did not increase any more (Fig. 9).

7. Effects of magnesium and manganese ion on B₁₂ uptake to brush borders

As shown in Figs. 10 and 11, IF-mediated B₁₂ uptake to brush borders (B) decreased on addition of manganese ion to the incubation medium containing calcium ion. In the medium containing 10 mM CaCl₂, the uptake decreased to about two-thirds by addition of MnCl₂ at the concentration of 20 mM (Fig. 10).

Fig. 9. $^{57}\text{Co-B}_{12}$ uptake to EDTA-treated brush borders incubated with IF-B $_{12}$ at 37°C for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing various concentrations of CaCl $_2$.

Fig. 10. $^{57}\text{Co-B}_{12}$ uptake to EDTA-treated brush borders incubated with IF-B $_{12}$ or B $_{12}$ alone at 37°C for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing (A) 10 mM CaCl $_2$, (B) 10 mM CaCl $_2$ and 20 mM MgCl $_2$, or (C) 10 mM CaCl $_2$ and 20 mM MnCl $_2$.

In the medium containing 2.5 mM CaCl $_2$, the inhibition by manganese ion began to occur at as low as a concentration of 1 mM of MnCl $_2$, and at a concentration of 20 mM MnCl $_2$, the uptake decreased almost to half of that in manganese-free medium (Fig. 11). Magnesium ion did not influence the uptake to brush borders (B) in calcium-containing medium (Figs. 10 and 11).

The uptake to brush borders (A) incubated in calcium-free medium was inhibited more conspicuously by addition of manganese ion (Fig. 12). Moreover, the uptake was inhibited by addition of magnesium ion, though the inhibitory

Fig. 11. ⁵⁷Co-B₁₂ uptake to EDTA-treated brush borders incubated with IF-B₁₂ at 37°C for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing 2.5 mM CaCl₂ and various concentrations of MgCl₂ or MnCl₂.

Fig. 12. ⁵⁷Co-B₁₂ uptake to non-treated brush borders incubated with IF-B₁₂ at 37°C for 1 hr in 0.01 M Tris-HCl buffer (pH 7.4) containing various concentrations of MgCl₂ or MnCl₂.

effect was much lower than that by manganese ion.

DISCUSSION

GLASS *et al.* (8) postulated that an intramural "intestinal B₁₂-acceptor" played the role in absorption of B₁₂ similar to that of apoferritin in iron absorption. This hypothesis was based on the fact that the efficiency of B₁₂ absorption de-

creased with the dose of administration. They felt that the absorption of B_{12} in the intestinal wall would cease with saturation of B_{12} acceptor, and that this might explain the regression of efficiency of B_{12} absorption on increase of the dosage.

GRÄSBECK and NYBERG (30) reported that the malabsorption of B_{12} in the patient with steatorrhea was normalized by the administration of calcium. They also observed that the B_{12} absorption was inhibited by the administration of EDTA in man, and that the absorption was preserved when calcium was administered simultaneously with EDTA. From the results it has been considered that the IF-mediated B_{12} absorption requires calcium ion and that the inhibited absorption in the patient of steatorrhea is attributable to the removal of calcium by forming insoluble soap with fatty acid. Therefore, the administration of calcium did not bring about recovery in a patient with pernicious anemia in whom IF or its activity was lacking (31).

HERBERT (32) observed that the rat liver slice uptake $^{60}\text{Co-B}_{12}$ by the incubation with hog IF but that no uptake occurred when calcium ion in the incubation medium was replaced by potassium ion. He also reported (10) that calcium ion intensified the B_{12} uptake to everted sacs of rat small intestine. Similar results were obtained in the experiment with homogenates of intestinal mucosa from rat (20). OKUDA (16, 33) studied the IF-mediated B_{12} absorption employing the intestinal loop technique and demonstrated that the pretreatment of the loop with EDTA or the addition of EDTA to the incubation medium completely abolished the enhancement of absorption by stomach extract.

In our experiments, brush borders treated with EDTA exhibit no uptake in calcium-free medium. But when incubated in a medium containing as small a concentration of CaCl_2 as 0.3 mM, the uptake reaches to maximal level. Moreover, the uptake to brush borders without EDTA-treatment occurs even in calcium-free medium. It is considered that calcium ion remaining in the tissue is able to enhance IF-mediated attachment of B_{12} to brush borders. Recently, FUJII (34) demonstrated that calcium ion was included in the receptor-IF- B_{12} complex by use of radioactive ^{40}Ca . Calcium ion in the tissue in our experiment might have been already bound to the receptor site of microvillous membranes of brush borders. And the B_{12} uptake to brush borders would seem to reach maximum when the receptor is saturated with calcium ion.

COOPER *et al.* (19) demonstrated that the IF-mediated B_{12} uptake to everted sacs of guinea pig small intestine was not inhibited by the removal of calcium and magnesium ion from the incubation medium. But addition of EDTA to the medium at a concentration of 0.09 mM inhibited the uptake. The inhibited uptake by 0.09 mM EDTA was recovered by the addition of 0.47 mM of calcium or 0.65 mM of magnesium, but the inhibition with 0.18 mM EDTA was not recovered upon addition of magnesium.

HERBERT and CASTLE (20) reported that the B_{12} uptake to everted sacs of rat small intestine was abolished by the removal of calcium and magnesium ion from

the incubation medium of KRB. But the removal of calcium ion alone did not abolish the uptake completely.

OKUDA and SASAYAMA (21) demonstrated that the inhibited B₁₂ absorption in man was recovered by administration of Ca²⁺, Mg²⁺ or Sr²⁺, but not by administration of Zn²⁺, Fe²⁺ or Fe³⁺.

CARMEL *et al.* (23) studied the B₁₂ uptake to human ileum homogenates in Krebs-Ringer-Tris buffer. The removal of magnesium alone did not abolish the uptake, but the removal of calcium alone abolished it in three of four normal gastric juices tested.

From these results it was suggested that magnesium could be partially substituted for calcium or that either cation was effective. But there are some discrepancies in these experiments. As Carmel mentioned, it was probable that "endogenous Ca²⁺ and Mg²⁺ in the gastric juice and gut homogenate accounted for the discrepancies in some cation omission experiments."

In this report, brush borders are treated with EDTA to avoid the effect of endogenous tissue-bound cation especially calcium. The uptake is not seen when incubated in a medium containing magnesium ion alone. This result indicates that magnesium ion can not be substituted for calcium ion.

There are no reports of the inhibitory effect of manganese ion on the intestinal absorption of vitamin B₁₂. As shown in this paper, manganese ion markedly inhibits the B₁₂ uptake to brush borders of rat intestine. The inhibitory effect is more conspicuous in the medium containing lower concentration of CaCl₂. And the uptake is most conspicuously inhibited when non-treated brush borders are incubated in calcium-free medium. It is suggested that the inhibitory effect of manganese might be related to calcium ion, though it is not clear whether it is competitive.

Calcium ion is necessary for normal synaptic transmission at chemical synapses, and manganese ion is known to inhibit synaptic transmission. Transmitter release from synaptic nerve endings in cats (35) or from neuro-muscular junction of the toad (36) is inhibited by manganese ion. It is assumed that manganese ion inhibit the release of transmitter by competing with calcium ion which is bound to receptor sites and that the receptor has a higher affinity for manganese than for calcium. The transmitter release at the neuro-muscular junction of the toad is almost completely inhibited by addition of 1.4 mM manganese to the solution containing 1.8 mM calcium, and the endplate potential is scarcely seen (36). Magnesium ion also compete with calcium ion in excitation-secretion coupling (37-39), but manganese is a much more effective competitor than magnesium is.

Recently, KANNO (40) observed that the calcium-dependent amylase release from pancreatic acinar cells was inhibited by manganese and he thought that the inhibitory effect was caused by competing with calcium. It is interesting that a similar phenomenon can be observed in his secretion study and in our absorption study.

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