Effect of Divalent Cations and pH on Intrinsic Factor-Mediated Attachment of Vitamin B_{12} to Intestinal Microvillous Membranes

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ABSTRACT Calcium, but not other divalent cations, is required for optimal uptake of intrinsic factor-bound ^{57}Co-labeled cyanocobalamin (IFB_{12}) by microvillous membranes isolated from hamster ileal-absorptive cells. Chelation of divalent cations by disodium ethylenediaminetetraacetate (EDTA) promptly removes IFB_{12} previously attached to microvillous membranes. High concentrations of $CaCl_2$ or $MgCl_2$ also markedly inhibit membrane uptake of IFB_{12} and rapidly remove previously attached IFB_{12}. Similarly, reduction of pH to below 5.4 prevents membrane attachment of IFB_{12} and removes virtually all IFB_{12} already bound to microvillous membranes. The effects of calcium depletion, increased salt concentrations, and acidification on membrane uptake of IFB_{12} were completely reversible. These findings are consistent with the concept that the formation of calcium salt bridges is essential for attachment of IFB_{12} to the ileal-absorptive surface.

INTRODUCTION

Divalent cations and pH appear to play a fundamental role in the intestinal absorption of vitamin B_{12}. Orally administered ethylenediaminetetraacetate (EDTA) inhibits absorption of the vitamin in human subjects, while simultaneous administration of calcium salts reverses this effect (1, 2). Acidification of intestinal contents impairs vitamin B_{12} absorption in experimental animals (3, 4), and absorption of the vitamin tends to be diminished in clinical circumstances associated with reduced intraluminal pH (5–7). In vitro, chelation of divalent cations or acidification of the incubation medium blocks intrinsic factor (IF)[1]-mediated uptake of the vitamin by everted sacs of intestine or by intestinal mucosal homogenates (8, 9).

Such findings have led to the concept (4, 8) that divalent cations and an appropriate pH may be required for normal attachment of IF-bound vitamin B_{12} (IFB_{12}) to specific receptors on the absorptive surface of the ileum. However, previous investigations have been concerned with preparations of whole ileal mucosa, and the results do not directly demonstrate that the action of divalent cations or pH actually occurs at the absorptive cell surface. It is possible, for example, that chelation of divalent cations and acidification inhibit tissue uptake of the vitamin merely by inducing nonspecific disintegration of intestinal epithelial tissue. Indeed, diminished B_{12} uptake and extensive intestinal mucosal damage occurs when one instills EDTA into isolated loops of canine intestine (10) or when the small bowel of experimental animals (11) or patients (12) is exposed to acid.

We have previously shown that IFB_{12} attaches to specific receptor sites present in purified preparations of microvillous membranes isolated from the apical surface of hamster ileal-absorptive cells (13). The present report describes the effects of divalent cations and pH on this attachment.

METHODS

Isolation of microvillous membranes. As previously described in detail (13), virtually pure preparations of microvillous membranes were isolated from the distal half of hamster small bowel by the method of Eichholz and Crane

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[1] *Abbreviations used in this paper*: B_{12}, cyanocobalamin; IF, intrinsic factor; IFB_{12}, intrinsic factor-bound ^{57}Co-labeled cyanocobalamin; KRB, Krebs-Ringer bicarbonate buffer.

(14). Final microvillous membrane preparations were washed with Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, and stored as a centrifuged pellet at − 20°C until used in uptake experiments.

Preparation of IFB_{12}. Gastric juice was obtained from hamsters allowed water but no food for 24 hr. As previously described in detail (13), the pylorus was ligated and 1 ml of 10% Na_2CO_3 was injected into the stomach to neutralize gastric acid as it was being secreted. After 5 hr, the alkaline gastric contents were collected and adjusted to pH 7.0 with 0.1 N HCl. Cyanocobalamin labeled with ^{57}Co (SA of 16–20 mCi/mg obtained from E. R. Squibb & Sons, Princeton, N. J.) was added in excess of the previously determined vitamin B_{12}-binding capacity of the gastric juice (13). After incubation at room temperature for 30 min, 3–5 ml of this mixture of radioactive cyanocobalamin and neutralized hamster gastric juice was dialyzed at 4°C in VisKing cellophane bags against 3 liters of 0.15 M NaCl for 48 hr during which the dialysis fluid was changed twice. After dialysis, gastric juice-bound B_{12}-^{57}Co was diluted with 0.15 M saline to a concentration of 2.0 ng/ml and stored at − 20°C.

Attachment of IFB_{12} to microvillous membranes. Microvillous membrane suspensions were diluted with KRB so that 1 ml contained 0.8 mg of membrane protein as determined by the method of Lowry (15). In 25-ml Erlenmeyer flasks, 1 ml of suspended microvillous membranes was added to 1 ml of IFB_{12} containing 2.0 ng B_{12}-^{57}Co. In control flasks, the incubation mixture was brought to a volume of 5.0 ml with KRB, pH 7.4. In experimental flasks, the microvillous membranes were washed and resuspended in an appropriately modified buffer and the same buffer was added to bring the final volume to 5.0 ml. For these experiments the incubation buffer was modified by adding disodium EDTA, by changing the pH, or by altering the concentration of divalent cations. After incubation at room temperature for 30 min, an excess of cold KRB or appropriately modified buffer was added to the mixture, and the membranes were centrifuged at 4°C for 30 min at 27,000 *g*. The membranes were then washed in an excess of cold KRB, pH 7.4, recentrifuged, and assayed for radioactivity in a Packard automatic gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The sensitivity of the radioassay system and the specific activity of the labeled vitamin B_{12} allowed estimation of 2 pg of radioactive vitamin with a counting error of less than 2%.

Removal of IFB_{12} already attached to microvillous membranes. For these experiments, we used centrifuged pellets of microvillous membranes which had been incubated with IFB_{12} in KRB pH 7.4 as described above. In control test tubes, these pellets were resuspended in 4.0 ml of 0.15 M NaCl-$NaHCO_3$ buffer, pH 6.5. After 5 min at room temperature, an excess of buffer was added, the suspension was again centrifuged at 27,000 *g* for 30 min at 4°C, the supernate was removed, and the centrifuged pellet was assayed for radioactivity. In experimental tubes, the NaCl-$NaHCO_3$ buffer was appropriately modified by the addition of disodium EDTA, or by the addition of divalent cations. In experiments designed to determine the effects of pH, KRB at pH 7.4 served as the control buffer while the pH of the KRB in the experimental tubes was varied by the addition of HCl.

Examination of radioactivity removed from microvillous membranes. After ^{57}Co radioactivity had been removed from microvillous membranes, the supernate containing removed radioactivity was concentrated by ultrafiltration through VisKing casing (16). The characteristics of this radioactivity were compared with those of hamster IFB_{12} which had not been previously incubated with microvillous membranes. To obtain sufficient radioactivity for adequate examination, it was necessary to pool supernates from replicate experiments. Sephadex G-200 gel filtration was performed in 0.05 M phosphate buffer pH 7.5 using a reverse flow 2.5 × 100 cm column (16). Vertical electrophoresis on starch gel was carried out for 20 hr at 4°C and 130 v in borate buffer, pH 8.6 (17). In other experiments radioactivity removed from microvillous membranes was dialyzed against KRB pH 7.4 for 48 hr. Uptake of this radioactivity by freshly prepared microvillous membranes was then compared with uptake of hamster IFB_{12} not previously exposed to membranes.

RESULTS

Attachment of IFB_{12} to microvillous membranes. As was demonstrated previously (13), hamster IF consistently enhanced uptake of B_{12}-^{57}Co by microvillous membranes prepared from the distal half of hamster small bowel. In 32 control experiments in which incubations were performed in KRB, mean uptake of IFB_{12} was 233±48 (SD) pg/mg membrane protein, while uptake of free B_{12}-^{57}Co was only 54±2 pg/mg of membrane protein. Storage of membrane preparations at −20°C for up to 1 wk before use did not impair uptake of IFB_{12}.

Table I indicates the effects of various divalent cations on IFB_{12} attachment to hamster microvillous membranes. Microvillous membrane pellets washed in 0.5 mM disodium EDTA, centrifuged, and resuspended in saline-bicarbonate buffer containing no divalent cations failed to take up significant quantities of IFB_{12}. When calcium ions were added to the incubation medium, however, IFB_{12} attachment increased markedly and was the same as that observed in control experiments performed in KRB. On the other hand, no significant uptake was observed when magnesium ions were added. Although

TABLE I

Effect of Divalent Cations on IFB_{12} Uptake by Hamster Microvillous Membranes

Divalent cation	Amounts added	IFB_{12} uptake
	µmoles	*pg B_{12}/mg protein*
none	—	4±1*
Ca^{++}	10	257±38*
Mg^{++}	10	3±1*
Mn^{++}	10	32
Mn^{++}	500	51
Sr^{++}	10	5
Sr^{++}	500	4
Hg^{++}	10	8
Hg^{++}	500	4
Zn^{++}	10	6
Zn^{++}	500	3

* Mean ±SD of eight experiments.

small amounts of IFB_{12} were taken up by membranes in the presence of manganese ions, addition of strontium, mercury, or zinc failed to promote attachment of IFB_{12}. As shown in Fig. 1, only small amounts of Ca^{++} were required to bring about IFB_{12} uptake. Addition of only 0.25 μmoles of Ca^{++} restored membrane uptake of IFB_{12} to control levels while Mg^{++} in amounts up to 500 μmoles were without effect.

Fig. 2 summarizes the effect of pH on IFB_{12} uptake by microvillous membranes. When the pH of the KRB buffer was adjusted to 5.4, attachment of IFB_{12} to membranes was almost completely prevented. Above pH 5.6, however, small increases in pH markedly increased IFB_{12} uptake so that at pH 5.8 uptake was about 50% and at pH 5.9 nearly 80% of the values observed in control experiments. At pH 6.5 uptake was fully restored to control levels.

Attachment of IFB_{12} to hamster intestinal microvillous membranes was inhibited in the presence of high concentrations of the chloride salts of calcium, magnesium, sodium, and potassium (Table II). At a concentration of 0.05 M, approximately 50-fold greater than present in control flasks, calcium and magnesium salts did not impair uptake of IFB_{12}. When concentrations were increased to 0.1, 0.5, and 2.5 M, however, attachment of IFB_{12} was increasingly inhibited. In contrast, uptake was not at all impaired when the molarity of the incubating medium was increased to the same extent by mannitol. The most striking inhibition was observed with $CaCl_2$ and the effects of $CaCl_2$ or $MgCl_2$ were obviously greater than those of KCl or NaCl. When membranes previously exposed to high concentrations of $CaCl_2$ or $MgCl_2$ were washed in KRB to remove the excess salt, subsequent IFB_{12} uptake of these membranes was not different from control values.

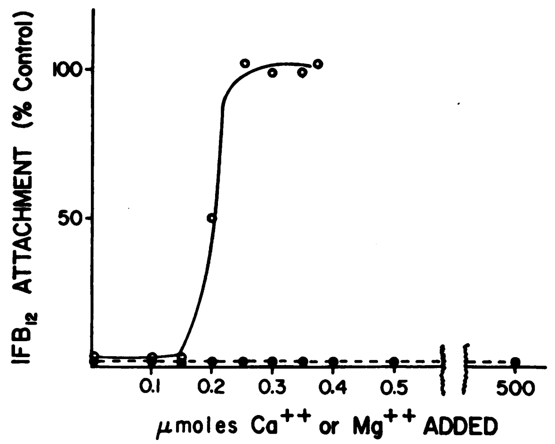

FIGURE 1 Effect of calcium (open circles, solid line) and magnesium (closed circles, broken line) on attachment of IFB_{12} to hamster intestinal microvillous membranes. Hamster microvillous membranes, washed with 0.5 mM disodium EDTA in 0.5 mM phosphate buffer, pH 6.5, were resuspended in saline-bicarbonate buffer to which was added $CaCl_2$ or $MgCl_2$. Each point represents the mean of two or four experiments.

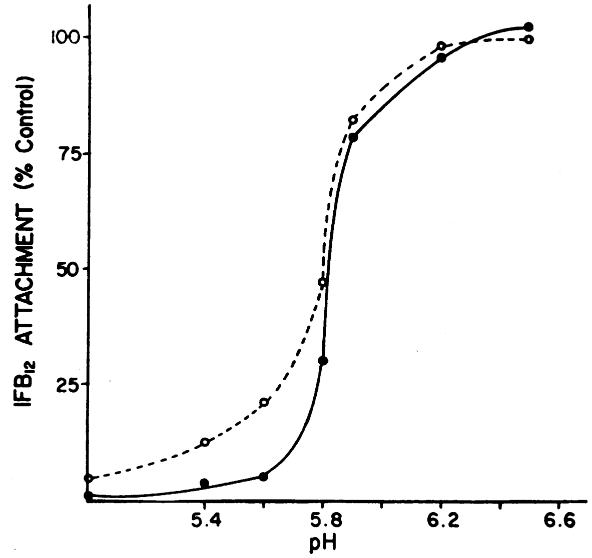

FIGURE 2 Effect of pH on attachment of IFB_{12} to hamster intestinal microvillous membranes. Control values were obtained with membranes maintained in KRB buffer, pH 7.4. In experimental flasks the pH of KRB buffer was adjusted by addition of HCl. Each point represents the mean of four experiments. The unbroken line indicates amounts of IFB_{12} taken up by microvillous membranes. The broken line indicates removal of IFB_{12} already attached to microvillous membranes.

Removal of IFB_{12} already attached to microvillous membranes. Membranes were incubated with IFB_{12} in KRB and then washed to remove any radioactivity not taken up by the membranes. When these membranes with attached IFB_{12} were subsequently exposed to disodium EDTA for 5 min, radioactivity was readily removed (Fig. 3). Addition of 5 μmoles of EDTA resulted in virtually complete removal of radioactivity from the

TABLE II

Effect of Salt Concentration on Attachment of IFB_{12} to Hamster Intestinal Microvillous Membranes

Salt concentration	IFB_{12} uptake			
	0.05 M	0.1 M	0.5 M	2.5 M
	*pg/mg membrane protein**			
$CaCl_2$	228±34	154±41	66±16	32±6
$MgCl_2$	237±47	246±28	108±22	59±14
NaCl	241±39	256±44	236±26	103±21
KCl	233±40	239±31	181±31	169±23

* Mean of two to five experiments ±SD.

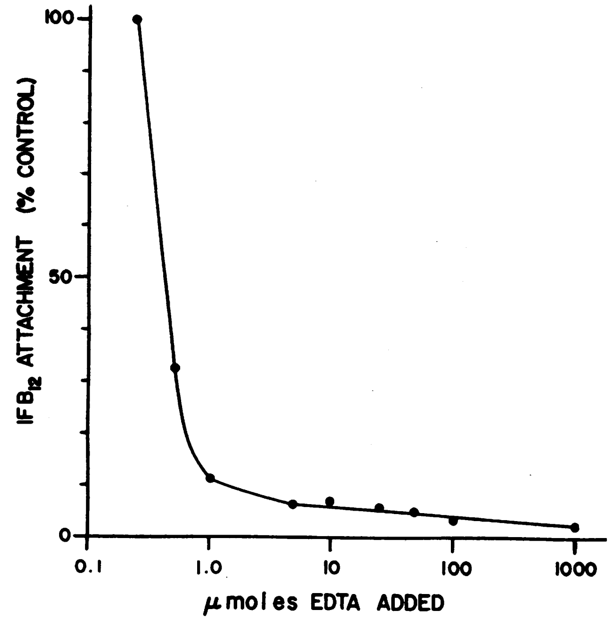

FIGURE 3 Effect of EDTA on attachment of IFB$_{12}$ to hamster intestinal microvillous membranes. Membranes previously incubated with IFB$_{12}$ in KRB buffer, pH 7.4 were washed with NaCl-NaHCO$_3$ buffer. Control values were obtained with membranes exposed for 5 min to NaCl-NaHCO$_3$ buffer while in experimental flasks membranes were exposed to buffer containing disodium EDTA. Each point represents the mean of two to eight experiments.

membranes. This effect of EDTA was completely prevented by the addition of calcium or magnesium ions. Membranes were also readily depleted of attached radioactivity when briefly exposed to acidified KRB. As shown in Fig. 2, the effects of pH on removing radioactivity previously attached to membranes were similar to the effects of pH on membrane uptake of IFB$_{12}$. When the pH of KRB was reduced to 6.5 no radioactivity was removed, while at pH 5.8 50% of membrane radioactivity was detached. Reduction of pH to 5.0 removed virtually all of the radioactivity from the membranes. Radioactivity was also detached when membranes were exposed to KRB modified by the addition of large amounts of calcium or magnesium. In the presence of 2.5 M $CaCl_2$ or $MgCl_2$ more than 80% of attached radioactivity was removed.

Fig. 4 demonstrated that the effects of EDTA and pH on the IFB$_{12}$ uptake by microvillous membranes were completely reversible. Membranes from which radioactivity had previously been detached by exposure to 5 μmoles of EDTA or to acidified KRB were washed in KRB. Subsequent uptake of IFB$_{12}$ by these membranes was not different from that observed during initial incubation experiments. Similarly, membranes depleted of radioactivity by exposure to high concentrations of $CaCl_2$ or $MgCl_2$ were fully capable of IFB$_{12}$ uptake when washed in KRB.

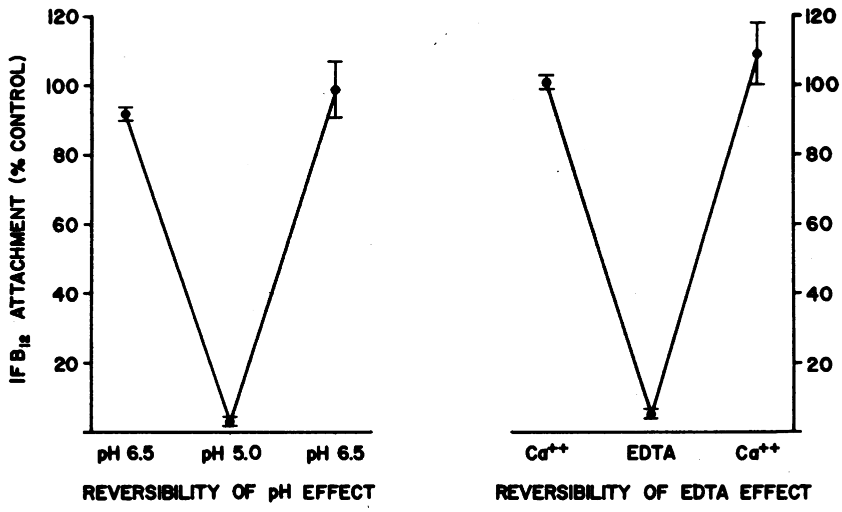

FIGURE 4 Reversibility of the effects of pH and EDTA on microvillous membrane uptake of IFB$_{12}$. When membranes were exposed for 5 min to KRB acidified to pH 5.0, virtually all previously attached IFB$_{12}$ was removed. When these same membranes were subsequently washed in KRB, pH 6.5, uptake of IFB$_{12}$ was not different from initial values. Similarly, exposure of membranes to disodium EDTA removed previously attached IFB$_{12}$. When these same membranes were subsequently washed in KRB containing calcium, uptake of IFB$_{12}$ was restored to initial levels. Each point represents the mean ±SD of four to eight experiments.

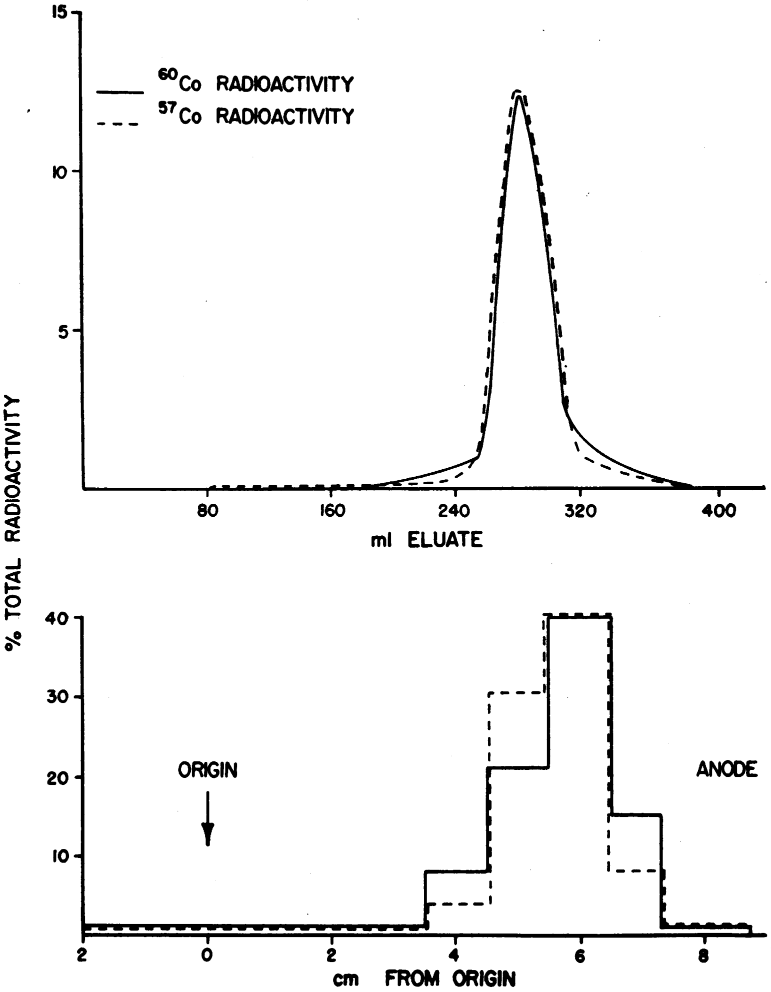

FIGURE 5 Comparison of ^{57}Co radioactivity removed from microvillous membranes with ^{60}Co-labeled cyanocobalamin bound to hamster IF. Upper panel shows filtration on Sephadex G-200. Lower panel shows starch gel electrophoresis. Microvillous membranes with previously attached ^{57}Co-labeled cyanocobalamin were exposed for 5 min to 0.5 mM EDTA. ^{57}Co radioactivity released from membranes (broken lines) was compared with ^{60}Co-labeled cyanocobalamin bound to hamster IF (unbroken lines).

Examination of radioactivity removed from microvillous membranes. Radioactivity detached from microvillous membranes by EDTA or by acid was not dialyzable. ^{57}Co radioactivity detached from membranes by EDTA was mixed with B_{12}-^{60}Co bound to hamster IF. When this mixture was subjected to gel filtration on Sephadex G-200, the elution pattern of the detached ^{57}Co radioactivity was not different from the pattern of IF-bound $B_{12}{}^{60}$Co (Fig. 5). Similar results were obtained when radioactivity removed from membranes by acidified KRB was compared with IF-bound B_{12}-^{60}Co. In addition, ^{57}Co radioactivity detached by EDTA or by acid had the same electrophoretic mobility in starch gel as radioactive B_{12} bound to hamster IF (Fig. 5). The ^{57}Co radioactivity removed from microvillous membranes by EDTA, by acid or by 2.5 M $CaCl_2$ or $MgCl_2$ was dialyzed against KRB. When this dialyzed radioactivity was subsequently incubated with freshly prepared microvillous membranes, tissue uptake of this radioactivity was not different from the uptake of IFB_{12} not previously exposed to microvillous membranes.

DISCUSSION

Although IF markedly enhances the uptake of cyanocobalamin (B_{12}) by various tissues in vitro, this enhancement requires the presence of divalent cations in the incubation medium. Removal of calcium ions from the medium greatly reduces hog IF-mediated uptake of B_{12}

by rat liver slices (18) or by everted sacs of rat ileum (8). Similarly, the absence of calcium ions prevents the effect of rat IF on B_{12} uptake by everted sacs of homogenates of rat ileum (8) as well as the effect of human IF on B_{12} uptake by everted sacs (9) and homogenates (19) of guinea pig distal bowel. In vivo, chelation of divalent cations by EDTA diminishes B_{12} absorption in humans (1) and rats (20) and inhibits uptake of the vitamin by isolated, perfused loops of rat ileum (4). Furthermore, EDTA consistently removes a significant proportion of radioactivity from everted intestinal sacs and isolated ileal loops (4, 9, 21) which have already taken up IF-bound radioactive B_{12}. All of these effects of EDTA are prevented by the addition of divalent cations.

Uptake of IFB_{12} is also modified by changes in pH. In vivo, acidification of the perfusion fluid prevents IF-mediated uptake of B_{12} by isolated, perfused loops of rat ileum (4). In vitro, lowering the pH of the incubation medium below pH 5.8 impairs uptake of IFB_{12} by everted sacs (8), homogenates (19), and isolated brush borders (13) of distal small bowel.

The present studies directly demonstrate that calcium ions and a pH greater than 6.5 are required for maximal attachment of IFB_{12} to the absorptive surface of hamster ileal cells. In the absence of divalent cations, IFB_{12} fails to attach to microvillous membranes isolated from these cells. Addition of small amounts of calcium promptly restores attachment to control levels, while magnesium and other divalent cations do not have this effect. In addition, chelation of divalent cations by small amounts of EDTA removes virtually all B_{12} radioactivity already attached to microvillous membranes. Similarly, reduction of pH completely prevents membrane uptake of IFB_{12} and removes previously attached radioactivity.

Although calcium ions and an appropriate pH appear to be crucial for the binding of IFB_{12} complex to its receptor on the microvillous membrane, neither acidification nor depletion of divalent cations damages irreversibly the IFB_{12} complex or the membrane receptor for IFB_{12}. When IFB_{12} is removed from microvillous membranes by EDTA or by reduction of pH, the membranes are fully capable of again taking up IFB_{12} after addition of calcium ions and readjustment of pH. Similarly, the radioactivity removed from membranes by acidification or by EDTA remains bound in a nondialyzable macromolecular complex which can not be distinguished from IFB_{12} by starch gel electrophoresis or by dextran gel filtration, and this complex attaches to microvillous membranes in the same way as does untreated IFB_{12}.

Thus calcium and an appropriate pH are crucial for binding IFB_{12} complex to isolated microvillous membranes, and neither calcium depletion nor acidification permanently damages the membrane receptor or the IFB_{12} complex. These findings localize an effect of calcium ions to the ileal-absorptive surface and are not consistent with the concept (10) that chelation of divalent cations with EDTA impairs intestinal uptake of vitamin B_{12} merely as a result of relatively nonspecific damage to ileal absorptive cells. In the present study, EDTA was used in a concentration less than that previously shown (10) to cause structural damage, the action of EDTA was demonstrable with a subcellular fraction of disrupted ileal cells and the effect of EDTA were completely reversible.

The precise mechanism by which calcium and hydrogen ions influence the binding of IFB_{12} to its membrane receptor remains unknown. Calcium forms salt "bridges" which link anionic groups, and the results of the present investigation support the concept that such anion-calcium-anion linkages may be involved in the attachment of IFB_{12} to ileal microvillous membranes. Calcium "bridges" would be disrupted by: (*a*) chelation of calcium by EDTA, (*b*) protonation of one or both anionic groups by acidification, or (*c*) neutralization of ionic charges by concentrated salt solutions. The effects of calcium depletion, acidification and high salt concentrations on calcium salt "bridges" should be completely reversible. Calcium could promote binding of IFB_{12} to the cell surface either by directly linking anionic groups on IF with those on the ileal receptor or by forming salt "bridges" which act to alter the conformation of the IFB_{12} complex or the membrane receptor. The present work establishes a primary role for calcium ions in the attachment of IFB_{12} complex to its receptor on the absorptive surface of the ileum, but further studies are required to delineate the mechanism of calcium action.

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