

MECHANISM OF INTRINSIC FACTOR ACTION IN EVERTED SACS OF RAT SMALL INTESTINE *

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Three decades have passed since Castle (1) and his associates demonstrated the existence of intrinsic factor, yet knowledge of its mechanism of action is still so limited that in 1958 two workers felt justified in concluding there was no such thing as intrinsic factor (2). In prior studies (3, 4), a mechanism involved in the enhanced uptake of vitamin B₁₂ by rat liver slices in the presence of hog intrinsic factor concentrate (HIFC) was demonstrated. This mechanism appeared to be dependent on the presence on liver slices of receptors for intrinsic factor, which in the presence of calcium would "take up" non-metabolically either free intrinsic factor or intrinsic factor bound to vitamin B₁₂. The present studies indicate the same mechanism may operate in the rat small intestine. A preliminary report of this work has been presented (5).

MATERIALS AND METHODS

The basic protocol for each experiment was that previously used in studies with rat liver slices (4). It consisted of two successive one hour incubations of everted sacs (6) of rat small intestine at 6° C. in air; the first with 0.5 mg. HIFC¹ in the Krebs-Ringer-Tris (4) incubation medium, the second with 0.01 μg. vitamin B₁₂-Co⁶⁰ added. Controls were run identically, except for exclusion of HIFC. Radioactivity retained in the mucosa of the everted sacs after three washings was determined in a well-type scintillation counter. Each sample was counted

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¹ Kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen, Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y. This preparation (No. WES671A) was clinically active in a daily oral dose of 5 mg., as determined by urinary excretion studies of the Schilling type (7).

for a minimum of 10,000 counts. Radioactivity was expressed as counts per minute per 10 cm. of small intestine length.

The sacs were prepared as follows: A healthy adult female rat of the Wistar strain was sacrificed. The small intestine was immediately removed by transecting the gut at the duodenal end, gently pulling it free of its mesenteric attachment, and transecting it again at the distal end of the ileum. The proximal 6 cm. was discarded. A No. 6 Courmand single lumen woven cardiac catheter² was slipped through the lumen of the small intestine, starting at the ileal end. When the catheter tip appeared at the duodenal end, a ligature was placed tightly over the ileal end to prevent passage of the base of the catheter. Continuous gentle traction on the tip of the catheter then brought about complete eversion of the small intestine, which was immediately placed in 0.9 per cent NaCl at 0° C. Adherent intestinal content was removed by washing in three changes of ice-cold 0.9 per cent NaCl. Starting at the duodenal end, the intestine was cut into 20 segments of equal length. The ends of each segment were closed with Schwartz straight temporary arterial intracranial clamps.³ No detectable adsorption of vitamin B₁₂-Co⁶⁰ onto these clamps was noted after the usual washings. The segments were placed sequentially into four beakers containing the standard incubation medium, so that segments numbered 1, 5, 9, 13 and 17 were in beaker No. 1, and so forth. Although only one experiment of each type is reported, each experiment was repeated at least twice for verification.

RESULTS

Simultaneous incubation. Sacs incubated in the standard medium with both HIFC and vitamin B₁₂-Co⁶⁰ added simultaneously showed no enhancement of vitamin B₁₂-uptake due to HIFC (Experiments 1 and 2, Table I). This was true at six different concentrations of HIFC, including the concentration (25 μg.) which produced a more than threefold enhancement of vitamin B₁₂-Co⁶⁰ uptake in the simultaneous incubation system with rat liver slices (4).

² Purchased from United States Catheter and Instrument Corp., 334 Bay Street, Glen Falls, N. Y.

³ Purchased from Storz Instrument Co., 4570 Audubon Avenue, St. Louis 10, Mo.

TABLE I

Effect of hog intrinsic factor concentrate (HIFC) on vitamin B₁₂-Co⁶⁰ uptake by everted sacs of rat small intestine under various experimental conditions

Experiment	Cpm/10 cm. of intestine		Experiment	Cpm/10 cm. of intestine	
	Total	Excess over control		Total	Excess over control
1. Simultaneous incubation			8. Effect of heated HIFC*		
Control	123	0	Control	95	0
500 µg. HIFC	104	-19	HIFC	502	407
50 µg. HIFC	88	-35	Heated HIFC	106	11
5 µg. HIFC	124	1	9. Effect of 5,6-dimethylbenzimidazole*		
2. Simultaneous incubation			Standard incubation		
Control	95	0	Control	124	0
37.5 µg. HIFC	89	-6	HIFC	469	345
25.0 µg. HIFC	88	-7	5,6-dimethylbenzimidazole added		
12.5 µg. HIFC	110	15	Control	117	0
3. Standard conditions*			HIFC	497	380
Control	126	0	10. Effect of CoCl ₂ *		
500 µg. HIFC	259	133	Standard incubation		
50 µg. HIFC	138	2	Control	106	0
5 µg. HIFC	129	3	HIFC	493	387
4. High HIFC concentrations*			CoCl ₂ added		
Control	103	0	Control	103	0
50,000 µg. HIFC	94	-9	HIFC	509	406
5,000 µg. HIFC	130	27	11. 5,6-dimethylbenzimidazole and CoCl ₂ *		
500 µg. HIFC	449	346	Standard incubation		
5. Calcium effect*			Control	115	0
Standard medium			HIFC	498	383
Control	196	0	5,6-dimethylbenzimidazole and		
HIFC	874	678	CoCl ₂ added		
Standard medium with sodium replacing calcium			Control	101	0
Control	301	0	HIFC	514	413
HIFC	680	379	12. Effect of pH 1.85*		
6. EDTA† effect*			Standard incubation		
3rd incubation without EDTA			Control	137	0
Control	98	0	HIFC	430	293
HIFC	510	412	First incubation at pH 1.85		
3rd incubation with EDTA			Control	141	0
Control	103	0	HIFC	302	161
HIFC	294	191	13. Effect of pH 1.85*		
7. 6° C. vs. 37° C.*			Standard incubation		
Standard incubation (6° C.)			Control	107	0
Control	133	0	HIFC	393	286
HIFC	597	464	Second incubation at pH 1.85		
Incubation at 37° C.			Control	109	0
Control	466	0	HIFC	227	118
HIFC	457	-9			

* Sequential incubation, *i.e.*, two successive one-hour incubations: the first with 0.5 mg. HIFC (unless otherwise stated) in the incubation medium, the second with 0.01 µg. vitamin B₁₂-Co⁶⁰ added.

† Disodium ethylenediaminetetraacetate dihydrate.

Sequential incubation. Sacs incubated first with 0.5 mg. of HIFC and then with vitamin B₁₂-Co⁶⁰ invariably showed a significant enhancement of the uptake of vitamin B₁₂-Co⁶⁰ (Table I). However, no clear enhancement was observed in the sequential incubation system when 10-fold or 100-fold higher or lower concen-

trations of HIFC were used (Experiments 3 and 4, Table I).

Effect of calcium-free medium. The enhancement of uptake produced by HIFC was markedly reduced in the absence of calcium (Experiment 5, Table I). The marked increase in the uptake of vitamin B₁₂-Co⁶⁰ by control tissues in the

absence of calcium may be due to increased cell permeability. The same phenomenon has been observed in studies with rat liver slices (3).

Effect of disodium ethylenediaminetetraacetate dihydrate (EDTA). After performance of the standard experimental procedure, sacs were incubated for a third hour in 10 ml. of Krebs-Ringer-Tris to which was added 2 ml. of 0.9 per cent NaCl either without disodium ethylenediaminetetraacetate dihydrate (EDTA) or containing 2 Gm. of the material per 100 ml. As noted in Table I, Experiment 6, the addition of EDTA brought about no change in control values, but markedly reduced the amount of vitamin B₁₂-Co⁶⁰ retained by sacs previously incubated in the presence of added HIFC.

Temperature. When the standard two incubations were performed at 37° C. instead of 6° C., the intestinal sac mucosa became pale and friable; microscopic examination⁴ demonstrated destruction of the mucosa. Such sacs showed very high control uptake and no enhancement by HIFC (Experiment 7, Table I).

Heating HIFC. The addition of HIFC which had been heated in a water bath at 100° C. for 15 minutes resulted in no enhancement of uptake of vitamin B₁₂-Co⁶⁰ (Experiment 8, Table I).

Effect of 5,6-dimethylbenzimidazole⁵ and CoCl₂. Neither 5,6-dimethylbenzimidazole nor CoCl₂ interfered with the uptake of vitamin B₁₂-Co⁶⁰ by everted sacs of rat small intestine either without or with preincubation with HIFC (Experiments 9, 10 and 11, Table I). This was true when either or both agents were added to the incubation medium just before the start of the second incubation and prior to the addition of vitamin B₁₂-Co⁶⁰. The amount of 5,6-dimethylbenzimidazole added was 1 μg. dissolved in 1 ml. of 0.9 per cent NaCl (producing a ratio of 100 units of 5,6-dimethylbenzimidazole to 1 unit of vitamin B₁₂-Co⁶⁰ by weight). The amount of CoCl₂ added was 1 ml. of 0.11 M solution.

Effect of incubation at pH 1.85. Performance of either the first or the second incubation at pH 1.85 instead of pH 7.5 had no effect on uptake

⁴ Microscopic preparations made through the courtesy of Dr. H. M. Zimmerman, Department of Pathology, Montefiore Hospital.

⁵ Kindly provided by Dr. K. Folkers, Merck Sharp & Dohme Research Laboratories, Rahway, N. J.

of control sacs but reduced the enhancement of vitamin B₁₂-Co⁶⁰ uptake normally observed after incubation with HIFC (Experiments 12 and 13, Table I).

DISCUSSION

A previous study (8) demonstrated that HIFC enhanced vitamin B₁₂ uptake by open segments of rat small intestine. Since both the mucosal and serosal surfaces were exposed to the incubation medium, such open segments were not suited to study of the mechanism of intrinsic factor action on the mucosal surface; results were complicated by serosal surface vitamin B₁₂ uptake which markedly elevated control values. This problem was eliminated in the present study by using closed everted sacs of rat small intestine (6). Only the mucosal surface of such sacs is exposed to the incubation medium.

The results of this study with everted sacs of rat small intestine are strikingly similar to the results of prior studies with rat liver slices (3, 4). In both cases, enhancement of vitamin B₁₂-Co⁶⁰ uptake by HIFC is most marked in a sequential incubation system (*i.e.*, a system of incubation with HIFC alone followed by a second incubation with vitamin B₁₂-Co⁶⁰ alone). In both cases this enhancement is observable in the cold, is maximal in the presence of adequate calcium, and is reversible to a significant degree by EDTA. In a calcium-free medium, no enhancement of vitamin B₁₂ uptake attributable to intrinsic factor occurs on liver slices, but a definite enhancement is still observable with everted sacs of small intestine. This may be due to difficulty in washing the everted sacs free of calcium prior to incubation.

That the increase in the mucosal uptake of vitamin B₁₂-Co⁶⁰ after preincubation with HIFC is due to intrinsic factor is suggested by the facts that: 1) the increase is abolished by preheating the HIFC at 100° C. for 15 minutes, a process which destroys intrinsic factor activity (9); 2) performance of either incubation at pH 1.85 markedly reduces the enhancement of the uptake of vitamin B₁₂-Co⁶⁰ otherwise observable (and no enhancement occurs if both incubations are performed at pH 1.85 [10]). (At this pH the effect of intrinsic factor in enhancing vitamin B₁₂ absorption from the rat intestine *in vivo* is

reduced [11]. It is not yet clear how much of the reduction is due to intrinsic factor instability at pH 1.85, and how much may be due to instability of vitamin B₁₂-intrinsic factor or intrinsic factor-receptor bonds at this pH.); 3) 5,6-dimethylbenzimidazole, either alone or in combination with CoCl₂, does not affect the binding by the system of vitamin B₁₂-Co⁶⁰, as it does not interfere with intrinsic factor action in human subjects (12). This latter point may suggest that in the sac formed by the everted small intestine of the rat, as in the small intestine of the human subject, the 5,6-dimethylbenzimidazole portion of cyanocobalamin, as well as the cobalt portion, may not be involved in the interaction with intrinsic factor. Of course, although these substances *as such* did not interfere with the uptake of vitamin B₁₂, it is possible that *as portions of the cyanocobalamin molecule* they may be involved in the interaction with intrinsic factor.

These findings would suggest that the mechanism of intrinsic factor action may be the same in the small intestine as in the liver slice. It may be postulated that intrinsic factor has the ability on the one hand to bind vitamin B₁₂ (9) and on

the other to attach to liver or intestine receptors in the presence of calcium. Possession of these two properties simultaneously may be unique to intrinsic factor. Heparin, chondroitin sulfate and orosomucoid do not increase vitamin B₁₂-Co⁶⁰ uptake by everted sacs of rat small intestine (10), as they did not increase vitamin B₁₂-Co⁶⁰ uptake by rat liver slices (3, 4). This would indicate that enhancement of vitamin B₁₂ uptake is not a nonspecific property of any mucopolysaccharide or mucoprotein.

The concept of an intestine receptor for intrinsic factor fits all the facts which led Wallerstein, Harris, Schilling and Castle (13) to "infer that the fundamental action of intrinsic factor is on a superficially located hypothetical structure or chemical process in the wall of the upper small intestine"; and led Glass, Boyd and Stephanson (14) to postulate the existence of an "intestinal vitamin B₁₂ acceptor." In addition, it allows of explanation of the control of absorption of physiologic amounts of vitamin B₁₂ in terms of a single mechanism at the cell surface, rather than of separate mechanisms at the mucosal membrane and intramurally.

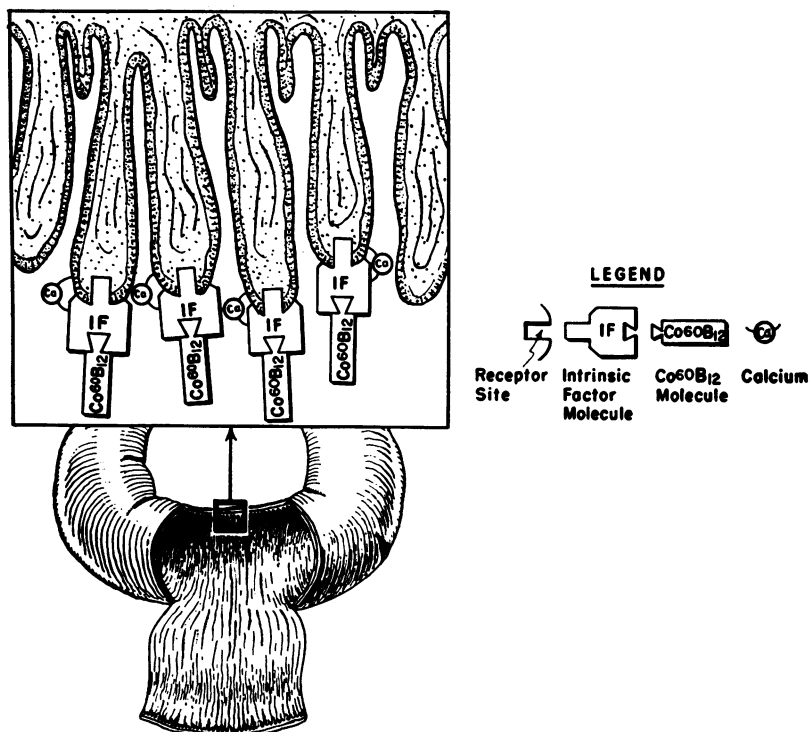


FIG. 1. OPTIMAL INTRINSIC FACTOR CONCENTRATION

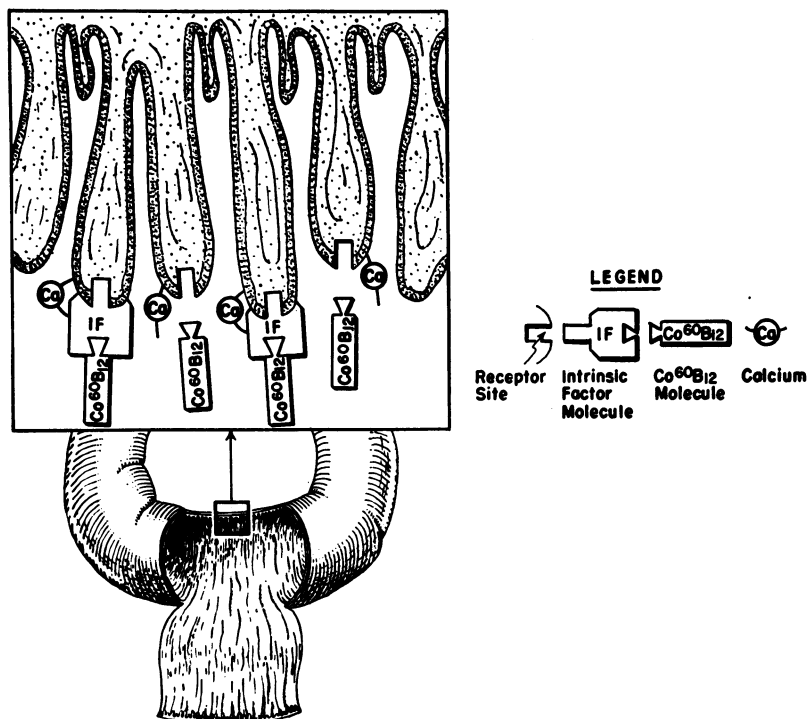


FIG. 2. HALF OPTIMAL INTRINSIC FACTOR CONCENTRATION

This formulation of the role of intrinsic factor in the first step in the absorption of physiologic amounts of vitamin B₁₂ is schematically depicted in Figures 1, 2 and 3. Figure 1 pictorially represents the ideal situation: an identical number of receptor sites, intrinsic factor molecules and vitamin B₁₂-Co⁶⁰ molecules. Maximal absorption of vitamin B₁₂ would occur in this optimal situation. In Figure 2, the decreased uptake of vitamin B₁₂ in the presence of an inadequate amount of intrinsic factor is portrayed. Figure 3 indicates the way the hypothesis of "receptor sites for intrinsic factor" may be used to explain the observed phenomenon *in vivo* (15-17) of decreased vitamin B₁₂ absorption in the presence of large amounts of intrinsic factor concentrates. When there are more molecules of intrinsic factor than there are receptor sites or molecules of vitamin B₁₂, a portion of the receptor sites will be occupied by intrinsic factor molecules which are not binding vitamin B₁₂. Since the excess of intrinsic factor saturates the receptor sites, that vitamin B₁₂ which remains in the lumen of the intestine, bound to intrinsic factor which has no available receptor site to go to, is lost in the feces.

It must be emphasized that alternative explanations for the inhibitory effect of large amounts of HIFC must be considered. These include: 1) possible contamination of HIFC preparations with nonintrinsic factor vitamin B₁₂ binding materials; 2) possible presence in HIFC preparations of inhibitors of intrinsic factor action. Neither of these possibilities can be ruled out until intrinsic factor is isolated in pure form. The most potent HIFC preparation available (No. WES655)⁶ inhibited vitamin B₁₂ uptake by everted sacs of rat small intestine when used in large quantity (10), but even this preparation can not be considered free of contaminants.

Whether this schema is an accurate depiction of the first stage of vitamin B₁₂ absorption *in vivo* remains to be determined. Preliminary observations, using sodium phytate to "tie up" calcium in the intestine of human subjects, suggest that the mechanism of intrinsic factor action may indeed be calcium-dependent (18). In other stud-

⁶ Kindly provided by Dr. W. L. Williams and Dr. L. Ellenbogen. This preparation was clinically active in a daily oral dose of 1 mg., as determined by urinary excretion studies of the Schilling type (7).

ies of the relationship of results in the *in vitro* system here reported to those *in vivo*, it was found that the maximal effect of HIFC occurs in those everted sacs from the middle two quarters of the small intestine (10); the maximal effect of intrinsic factor in the living rat is also in the middle quarters of the small intestine (19). The reason for discarding the proximal 6 cm. of small intestine in all the everted sac experiments reported here was that no effect of HIFC was demonstrable in that portion of the gut.

A further parallel of the *in vitro* system with the *in vivo* situation is the demonstration of two separate mechanisms of vitamin B₁₂ uptake in both. One mechanism is intrinsic factor-dependent, and plays the major role in absorption of physiologic amounts of vitamin B₁₂ (20, 21). It is this mechanism with which this report is mainly concerned. The other mechanism appears to be a "mass action phenomenon," possibly diffusion, and is operative in bringing about the absorption of small amounts of vitamin B₁₂. This latter mechanism is most readily observable in the human subject after the oral administration of supraphysiologic amounts of vitamin B₁₂

(20, 21). In the everted sac of rat small intestine, it may be this mechanism which produces the vitamin B₁₂ uptake observed in controls.

The failure to observe enhanced vitamin B₁₂-Co⁶⁰ uptake by everted sacs of rat small intestine after incubation with large amounts of HIFC, or after simultaneous incubation with amounts of HIFC which produced marked enhancement of liver slice vitamin B₁₂ uptake, is not yet explained. The former may be explainable by "trapping" of large amounts of HIFC between the intestinal villi, to be released during the subsequent incubation with vitamin B₁₂-Co⁶⁰ and then to compete for the vitamin with the intrinsic factor on receptor sites. The latter may be explainable by the presence of fewer free receptors on the intestinal wall than on the liver slice. This is suggested by the fact that enhancement of vitamin B₁₂ uptake by HIFC is not as many times more than control values for intestine sacs as for liver slices. It is possible that many receptor sites on the rat intestinal wall are occupied by rat intrinsic factor binding vitamin B₁₂ taken in the rat's diet prior to sacrifice. Studies with everted sacs of small intestine from

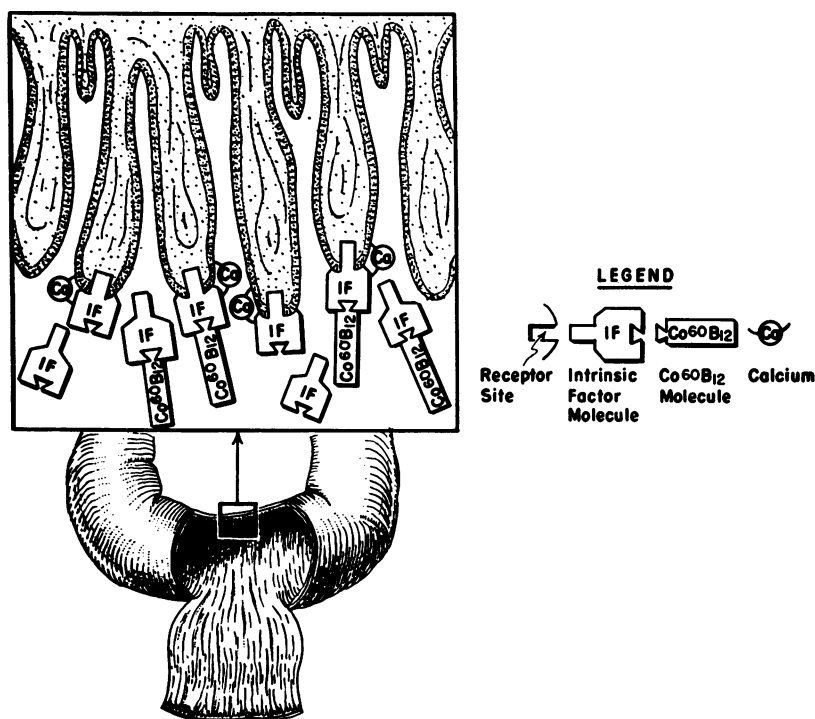


FIG. 3. TWICE OPTIMAL INTRINSIC FACTOR CONCENTRATION

gastrectomized rats may provide useful information in this connection.

Finally, the demonstration that hog intrinsic factor concentrate will enhance vitamin B₁₂ uptake by rat small intestine further weakens the indirectly (22) and directly (17) attacked concept of species specificity of intrinsic factor as a phenomenon of major importance. Data previously reported (8) would suggest the interesting speculation that perhaps an organ specificity may exist for intrinsic factor and may be of greater significance than the species specificity.

SUMMARY

Vitamin B₁₂ uptake by everted sacs of rat small intestine is enhanced markedly after preincubation with hog intrinsic factor concentrate (HIFC). This enhancement occurs at 6° C., is maximal in a calcium-containing incubation medium, and is reversible to a significant degree by disodium ethylenediaminetetraacetate dihydrate (EDTA). Procedures which interfere with intrinsic factor action, such as heating at 100° C. or incubating at pH 1.85, eliminate or reduce this enhancement.

The close similarity of these findings to those previously obtained with rat liver slices is discussed. The hypothesis is presented that the first step in the absorption of physiologic amounts of vitamin B₁₂ by the small intestine is binding of the vitamin to intrinsic factor and binding of intrinsic factor to intestine receptors (in the presence of calcium). It is suggested that possession of these two binding properties simultaneously may be unique to intrinsic factor.

The uptake of vitamin B₁₂ by everted sacs of rat small intestine in the absence of HIFC may be the *in vitro* parallel of a nonintrinsic factor-dependent mechanism of vitamin B₁₂ absorption observed *in vivo* and operative primarily in the presence of supraphysiologic amounts of vitamin B₁₂.

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