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Victor Herbert, William B. Castle

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Research Article

DIVALENT CATION AND pH DEPENDENCE OF RAT INTRINSIC FACTOR ACTION IN EVERTED SACS AND MUCOSAL HOMOGENATES OF RAT SMALL INTESTINE*

By VICTOR HERBERT and WILLIAM B. CASTLE

(From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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In 1959 it was reported in these pages (1) that hog intrinsic factor concentrate enhanced the uptake of radioactive vitamin B_{12} (Co^{60}-B_{12}) by everted sacs of rat small intestine in the cold. This enhancement was significantly reduced in the absence of calcium and was reversible to a significant degree by disodium ethylenediamine tetraacetate dihydrate (EDTA) (1), suggesting that intrinsic factor action was calcium-dependent. Disodium EDTA reduced the enhancing effect on radio-B_{12} uptake of rat intrinsic factor in loops of small intestine in a living rat; calcium EDTA was without such inhibitory effect (2). Other workers, however, threw doubt on the calcium dependence of intrinsic factor action. They reported that gastrectomized rats fed radio-B_{12} bound to rat gastric juice plus large amounts of disodium EDTA (3, 4), and normal rats fed radio-B_{12} plus disodium or tetrasodium EDTA (5), had normal radio-B_{12} absorption. These findings, taken with the fact that the *in vitro* studies did not demonstrate total loss of the effect of intrinsic factor on depletion of calcium, left the concept of the calcium dependence of intrinsic factor action in doubt.

The present report presents the results of a study aimed at resolving this uncertainty, and also at strengthening the evidence (6, 7) for the pH dependence of intrinsic factor action.

MATERIALS AND METHODS

Co^{60}-labeled vitamin B_{12} and Co^{57}-labeled vitamin B_{12}.[1] The Co^{60}-labeled vitamin B_{12} had a specific activity of approximately 1 μc per μg, and the Co^{57}-labeled vitamin B_{12} had a specific activity of approximately 5 μc per μg. The pH-dependence experiments using everted sacs were performed with Co^{57}-labeled vitamin B_{12}; all other experiments were performed with Co^{60}-labeled vitamin B_{12}.

Rat intrinsic factor concentrate (RIFC) was prepared as previously described (2).

Rat small intestine everted sac system. The homologous system (RIFC, everted sacs of rat small intestine) used by Strauss and Wilson (8–10) was employed.

Nonfasting male Sprague-Dawley rats were sacrificed. The small intestine was transected at the pylorus and 25 to 50 ml of 0.9 per cent NaCl was run through it to rinse out particulate content. The small intestine was then stripped free of its mesenteric attachment, transected at its ileal end and everted on a stainless steel rod, 112 cm long and 1.5 mm in diameter, with rounded ends. Beginning with its lower end, the rat small intestine was slipped over the rod until a silk ligature could be placed tightly over the ileal end of the intestine, and held in place by a groove in the rod 2 mm from its end. The intestine was gently and rapidly everted on itself. It was then washed by dipping repeatedly into each of two changes of 200 ml saline, and placed in a petri dish containing 0.9 per cent saline at room temperature.

Individual sacs approximately 4 cm long were prepared as described by Wilson and Wiseman (11). After being ligated at each end and slightly distended with Krebs-Henseleit bicarbonate (12) containing 200 mg per 100 ml glucose, each sac was dropped into a separate 50-ml Erlenmeyer flask containing 5 ml incubation medium plus 5,000 $\mu\mu$g of radioactive vitamin B_{12} in 0.5 ml of 0.9 per cent saline with or without added RIFC. The amount of RIFC used was prepared from 1/100 of a rat stomach, diluted with 0.9 per cent NaCl to a volume of 0.5 ml. This amount of rat stomach was chosen because it produced near maximal enhancement of vitamin B_{12} uptake in this everted sac system. Indeed, tenfold lower or higher amounts of RIFC produced markedly less enhancement, and still larger amounts inhibited radio-B_{12} uptake (data not shown).

Immediately after placing each filled everted sac in the medium, the flask was gassed for 60 seconds with 95 per cent O_2 and 5 per cent CO_2, sealed with a rubber stopper, and shaken (96 cycles per minute) at 37° C for 1 hour. At the end of this period the sac was removed and rinsed through three changes of 0.9 per cent saline. The ligated ends were then cut off, the sac cut open lengthwise and gently blotted with toilet tissue, weighed,

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[1] Kindly provided by Drs. C. Rosenblum, N. Ritter and E. Alpert of Merck Sharp & Dohme Research Laboratories, West Point, Pa.

and the quantity of its radioactivity determined in a well-type scintillation detector, for which the magnitude of the background averaged 330 counts per minute. In this detector the Co^{60}-labeled vitamin B_{12} registered approximately 1 count above background per minute per $\mu\mu g$, and the Co^{57}-labeled vitamin B_{12} registered approximately 4 counts above background per minute per $\mu\mu g$.

In all experiments with everted sacs, except those in which the site of intrinsic factor action was studied, the top and bottom 20 per cent of the rat small intestine was discarded and sacs were prepared from the remainder, beginning at its top. This was done because of the minimal-to-absent action of RIFC at top and bottom of the rat small intestine. Each experiment was repeated at least three times in order to ensure validity. Sac length was measured by placing a ruler alongside the unstretched gut, which had been allowed to retract while in 0.9 per cent NaCl at room temperature in a large petri dish.

Rat small intestine mucosal homogenate system (rat gut homogenate system). Nonfasting 200 to 300 g male Sprague-Dawley rats were sacrificed. The small intestine was transected at the pylorus, stripped free of its mesenteric attachment, and transected at its ileal end. It was then placed on a long strip of Parafilm,[2] measured, and the top and bottom 20 per cent discarded. The remaining small intestine was everted, washed free of adherent intestinal contents by dipping repeatedly into each of two changes of 0.9 per cent NaCl, and cut with scissors into roughtly 7- to 8-cm segments. Each segment was then scraped free of mucosa with two glass slides (one holding the gut in a fixed position, the other used for scraping). The mucosal scrapings from two rats were combined and placed in 25 ml of 0.9 per cent NaCl in a 50-ml beaker, shaken vigorously by hand for 10 seconds and then homogenized for 30 seconds in a Waring Blendor. Then, after being centrifuged for 10 minutes at 3,000 rpm, the liquid intermediate phase was discarded, leaving together for final suspension both the particulate material that initially floated above the liquid phase and that which sedimented. Sufficient 0.9 per cent NaCl was added to this rat small intestine homogenate so that 1 ml of the resultant suspension of homogenate could easily be pipetted into each of a series of 50-ml Erlenmeyer flasks. The homogenate prepared from one rat intestine was sufficient for 8 to 14 such flasks.

In the experiments here reported, incubation with rat gut homogenate was performed as with everted sacs, except that the gas phase was air. Freshly prepared rat gut homogenate was used, although thawed frozen homogenate is equally useful in the "assay" of RIFC (data not presented). Incubation was followed by centrifugation for 10 minutes at 3,000 rpm. The liquid phase was then removed by pipet, followed by resuspension in and centrifugation from two washes in 10 ml of 0.9 per cent NaCl containing 10 mmoles $CaCl_2$. The quantity of radioactivity retained by the sedimented rat gut homogenate was then determined in a well-type scintillation detector.

In the studies of pH dependence of intrinsic factor action, the buffer used consisted of 9 parts Krebs-Ringer solution (13), brought to appropriate pH with 1 part 0.05 M Tris-(hydroxymethyl)aminomethane-acid maleate-NaOH buffer (14). Immediately after placing each everted sac in the medium, the flasks were gassed with 100 per cent O_2 and shaken at 37° C for one-half hour. The same incubation system was used for pH-dependence

[2] Marathon Co., division of American Can Co., Menasha, Wis.

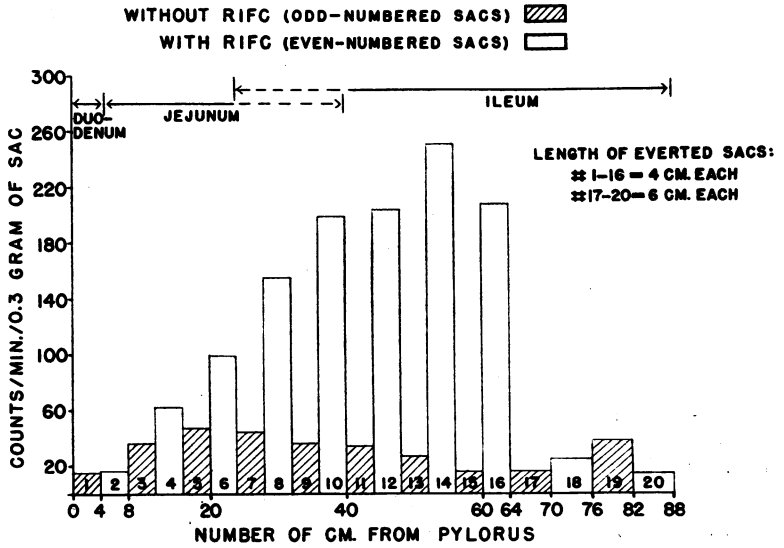

FIG. 1. ABSORPTION OF VITAMIN B_{12} AT VARIOUS LEVELS OF THE RAT SMALL INTESTINE *in vitro*, WITHOUT AND WITH ADDED RAT INTRINSIC FACTOR CONCENTRATE (RIFC).

TABLE I

*Divalent cation dependence of rat intrinsic factor concentrate (RIFC) in augmenting adsorption of Co^{60}-B_{12} by everted sacs * and mucosal homogenates of rat small intestine*

Incub. medium†	RIFC	cpm/ everted sac†	Excess counts over control sacs	cpm/ aliquot gut homogenate	Excess counts over control homogenate
Standard	0	45		264	
Standard	+	168	123	665	401
Standard − Ca‡	0	72		246	
Standard − Ca‡	+	121	49	488	242
Standard − Mg‡	0	29		231	
Standard − Mg‡	+	190	161	588	357
Standard − (Ca + Mg)‡	0	73		60	
Standard − (Ca + Mg)‡	+	92	19	53	−7
Standard	0	19			
Standard	+	307	288		

* All sacs from midportion of small intestine and about 4 cm long.

† Standard medium is Krebs-Henseleit bicarbonate, containing 200 mg % glucose and 5,000 μμg Co^{60}-B_{12}, gassed with 95% O_2-5% CO_2 (pH 7.4).

‡ Each divalent cation deleted was replaced by an equivalent millimolar concentration of sodium.

studies with rat gut homogenate, except that the gas phase was air.

RESULTS

Site of intrinsic factor action in the rat small intestine. Figure 1 demonstrates that uptake of vitamin B_{12} mediated by RIFC is greatest in everted sacs from the midportion of the rat small intestine. A relatively high Co^{60}-B_{12} uptake by the teminal ileum in the absence of RIFC was observed in three of four separate rat intestines.

Divalent cation dependence of RIFC action. Table I demonstrates that RIFC does not appreciably enhance vitamin B_{12} uptake by everted sacs of rat small intestine when both calcium and magnesium are deleted from the medium. Deletion of calcium alone appears to reduce markedly the effect of RIFC. Deletion of magnesium alone does not. The finding that deletion of calcium from the incubation medium results in higher radio-B_{12} uptake by control sacs (without RIFC) has been previously noted (1). The calcium and magnesium dependence of RIFC action is also

TABLE II

*pH dependence of rat intrinsic factor concentrate in augmenting absorption of Co^{57}-B_{12} by everted sacs * of rat small intestine*

pH of buffered medium†				
Before inc.	After inc.	RIFC	cpm/ everted sac	Excess counts over control
7.1	6.3	0	123	
7.1	6.3	+	259	136
5.7	5.7	0	102	
5.7	5.7	+	44	−58
5.7	6.8‡	0	103	
5.7	6.7‡	+	299	196
7.1	5.7§	0	86	
7.1	5.7§	+	35	−51
7.1	6.7	0	78	
7.1	6.8	+	488	410

* All sacs as in Table I; each filled with 1.1 ml buffered medium at pH 7.1.

† Buffered external medium in each flask contained 5,000 μμg Co^{57}-B_{12} with a gas phase of 100% O_2.

‡ 0.085 ml 0.2 M NaOH had been added to buffer after first 15 minutes of incubation.

§ 0.2 ml Tris acid maleate had been added to buffer after first 15 minutes of incubation.

TABLE III

pH dependence of rat intrinsic factor concentrate in augmenting absorption of Co^{60}-B_{12} by mucosal homogenate of rat small intestine

pH of buffered medium*				
Before inc.	After inc.	RIFC	cpm/ aliquot gut homogenate	Excess counts over control homogenate
4.6	4.6	0	293	
4.6	4.6	+	156	−137
5.4	5.4	0	243	
5.4	5.4	+	135	−108
5.9	5.9	0	210	
5.9	5.9	+	231	21
6.5	6.5	0	229	
6.5	6.5	+	372	143
6.9	6.9	0	236	
6.9	6.9	+	409	173
7.4	7.4	0	214	
7.4	7.4	+	406	192
7.9	7.7	0	214	
7.9	7.7	+	463	249
8.4	8.2	0	214	
8.4	8.2	+	454	240
8.9	8.6	0	221	
8.9	8.6	+	529	308

* 5,000 μμg Co^{60}-B_{12} added to each aliquot of buffered medium. Incubation performed with air as gas phase.

demonstrable in rat small intestine mucosal homogenate (Table I).

pH dependence of RIFC action. Table II shows that RIFC enhances vitamin B_{12} uptake by everted sacs of rat small intestine at pH 7.1 but not at pH 5.7. RIFC previously kept at pH 5.7 for 15 minutes at 37° C enchances vitamin B_{12} uptake by everted sacs when the pH is raised to 6.8. The enhancing effect of RIFC at pH 7.1 is reversed to one of inhibition by lowering the pH to 5.7.

Table III indicates that the enhancing effect of RIFC on vitamin B_{12} uptake by rat small intestine homogenate increases stepwise as the pH is raised stepwise from 5.9 to 8.9.

DISCUSSION

The question of relevance to physiological fact arises with all *in vitro* systems, and can only be settled in each case by demonstrating that what occurs *in vitro* also occurs in physiological circumstances *in vivo*. It has been demonstrated *in vivo* (15, 16) that the major site of absorption of vitamin B_{12} in the rat is the midportion of the small intestine. Prior studies (9, 17), confirmed here (using the entire length of rat small intestine), demonstrate that intrinsic factor-mediated vitamin B_{12} absorption in everted sacs of rat small intestine is greatest in the same portion of the small intestine. This indicates anatomical and physiological similarities of the *in vitro* everted sac system to the *in vivo* system for the absorption of vitamin B_{12} in the rat. Moreover, in quantitating the sites of vitamin B_{12} absorption, the *in vitro* system excludes *in vivo* variations in the contents of the intestinal lumen, such as distally diminished amounts of intrinsic factor and radioactive vitamin B_{12} or local differences in pH.

The finding of calcium dependence of intrinsic factor action at 37° C in everted sacs of rat small intestine and in rat small intestine homogenate using homologous intrinsic factor also has a counterpart *in vivo* (2). In man, short-term (18, 19) or long-term (19, 20) treatment with calcium-chelating agents appears to inhibit vitamin B_{12} absorption and eventually to lower serum vitamin B_{12} levels (19, 20).

Calcium appears to be essential for binding of intrinsic factor to the hypothetical receptors on the small intestinal mucosa (1, 2). It is interesting that in Hydra, which requires bound calcium for the action of surface receptors, magnesium antagonizes rather than partially substitutes for the action of calcium (21). The finding that magnesium may partially substitute for calcium was foreshadowed by the evidence that magnesium EDTA was a less potent blocker of RIFC action than was disodium EDTA (2).

The studies of pH dependence here reported reinforce previous clinical (6) and experimental (7) findings *in vivo* and *in vitro*. They demonstrate that RIFC is unable to enhance vitamin B_{12} absorption at pH 5.7, but that a slight rise in pH to 5.9 or above allows such enhancement. The suppression of ionization of carboxyl groups on mucosal cell surfaces (22) or on mucoproteins (23) by low pH may be related to the dependence of RIFC adsorption on divalent cations such as Ca^{++} and Mg^{++}. At any rate, it appears that the ineffectiveness of RIFC at pH 5.7 is *not* due to damage to the molecule, since its enhancement ability is restored on again raising the pH. These findings suggest that a pH above 5.7 is required for intrinsic factor to attach to receptors on the intestinal mucosa, especially since the enhancing effect of RIFC on vitamin B_{12} uptake by everted sacs of rat small intestine appears to become inhibitory when the pH of the system is reduced to 5.7. This may be due to a combined effect: the low pH obliterates the RIFC enhancement and RIFC binds most of the vitamin B_{12} (2), leaving very little free in solution to diffuse into the gut mucosa.

Preliminary experiments (not shown) demonstrated that comparable enhancing effects with RIFC upon Co^{60}-B_{12} absorption could be demonstrated in either everted sacs or suspensions or homogenates of mucosal scrapings from the rat small intestine. The homogenate system possesses the advantage that it eliminates variations in the measurement or in the inherent activity of the mucosa of different everted mid-intestinal segments (Figure 1). Consequently, a single aliquot of the mucosal homogenate can serve as a control for a number of experimental flasks. In this respect the intestinal homogenate system has the advantages for RIFC studies that the liver homogenate system (24) possesses for studies (24, 25) of hog intrinsic factor concentrate activity. In the intestinal homogenate system, as in

the liver homogenate system, the intrinsic factor effect is observable at room temperature and at 3° C, as well as at 37° C (data not presented). Since each aliquot of intestinal homogenate is identical with every other aliquot, this system appears to provide a fairly quantitative assay for intrinsic factor activity. Unpublished studies (26) indicate that homogenate prepared from the bottom half of guinea pig small intestine may prove more useful for "assay" of human intrinsic factor activity than the everted sacs of guinea pig small intestine previously (10) suggested.

SUMMARY

1. Prior *in vivo* and *in vitro* findings that the midportion of the small intestine of the rat is the site of vitamin B_{12} absorption were confirmed *in vitro*.

2. Calcium is required for rat intrinsic factor concentrate (RIFC) adsorption to everted sacs of rat small intestine or to rat small intestine mucosal homogenate *in vitro*. Magnesium can partially substitute for calcium.

3. The enhancing effect of RIFC on vitamin B_{12} absorption occurs at pH 5.9 or higher. It is sharply pH dependent and at pH 5.7 is reversed to an inhibitory effect.

4. Small intestine mucosal homogenate is a useful tool for studies of intrinsic factor activity and the factors affecting it.

ADDENDUM

At the Second European Symposium on Vitamin B_{12} and Intrinsic Factor (Hamburg, Germany, August 2-5, 1961) (Proceedings to be published by Ferdinand Enke Verlag, Stuttgart) it was reported by Niewig, Abels, Vegter and Hellemans that sodium bicarbonate enhanced the decreased vitamin B_{12} absorption of two patients with pancreatic insufficiency, presumably by raising the low pH of the duodenal contents. Their results suggest that our findings in rats *in vitro* may be applicable to man *in vivo*.

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