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Research Article

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Characterization of Ileal Vitamin B_{12} Binding Using Homogeneous Human and Hog Intrinsic Factors

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ABSTRACT Elucidation of the mechanism of intrinsic factor (IF)-mediated vitamin B_{12} (B_{12}) binding to ileal binding sites has been hampered by the use of crude or only partially purified preparations of IF in previous studies. We have used homogeneous human IF and hog IF isolated by affinity chromatography to study [^{57}Co]B_{12} binding to ileal mucosal homogenates. The following observations were made: (*a*) Human IF-B_{12} and hog IF-B_{12} were bound to human, monkey, hog, dog, rabbit, mouse, hamster, and guinea pig ileal, but not jejunal, homogenates in amounts significantly greater than free B_{12} or B_{12} bound to five other homogeneous B_{12}-binding proteins; (*b*) only IF-mediated B_{12} binding was localized to ileal homogenates and was inhibited by EDTA; (*c*) values for the association constant (K_a) for the various ileal homogenates mentioned above and human IF-B_{12} and hog IF-B_{12} ranged from 0.3×10^9 M^{-1} to 13.0×10^9 M^{-1}. Apparent differences in the K_a for human IF-B_{12} and hog IF-B_{12} existed in most species; (*d*) the number of ileal IF-B_{12} binding sites per gram (wet weight) of ileal mucosa ranged from 0.3×10^{12} to 4.9×10^{12}. The same value was always obtained with human IF-B_{12} and hog IF-B_{12} for any given homogenate preparation; (*e*) 100-fold excesses of free B_{12} or human IF and hog IF devoid of B_{12} did not significantly inhibit human IF-B_{12} and hog IF-B_{12} binding to human and hog ileal homogenates.

These experiments performed with homogeneous IF indicate that: (*a*) gastric factors other than IF are not required for B_{12} binding to ileal IF-B_{12}-binding sites; (*b*) the mechanism of ileal IF-B_{12} binding is different from that of free B_{12} or of B_{12} bound to non-IF-B_{12}-binding proteins; (*c*) human IF and hog IF have different structures; (*d*) human IF-B_{12} and hog IF-B_{12} bind to the same ileal binding sites; and (*e*) human and hog ileal IF-B_{12} binding sites bind free B_{12} and human and hog IF devoid of B_{12} poorly, if at all.

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INTRODUCTION

In many animals, including man, the stomach synthesizes and secretes a glycoprotein known as intrinsic factor (IF)[1] that binds vitamin B_{12} (B_{12}) and facilitates the absorption of the vitamin in the distal small intestine. The mechanism of IF-mediated B_{12} absorption is not well understood, but a number of studies have demonstrated that IF facilitates B_{12} binding to ileal sacs (1–6), mucosal homogenates (4, 7–10), microvillus membrane preparations (11, 12), and to a component solubilized from ileal mucosa with Triton X-100 (13). IF-facilitated B_{12} binding of this type is dependent on pH, is inhibited by EDTA, and is postulated to represent the first step in the complex process by which B_{12} passes from the intestinal lumen into the portal blood.

Previous studies of IF-facilitated ileal B_{12} binding have employed crude or only partially purified preparations of IF, however, and for this reason a number of aspects of this phenomenon remain unclear. The areas of uncertainly include the following: (*a*) whether free B_{12} and IF devoid of B_{12} compete with the IF-B_{12} complex for binding to ileal IF-B_{12} binding sites (2, 7, 9, 11, 14, 15) or not (3, 6, 8, 16); (*b*) whether gastric factors other than IF are required for IF-B_{12} binding to ileal binding sites as has been suggested (15); and (*c*) whether B_{12}-binding proteins lacking IF activity in

[1] *Abbreviations used in this paper:* B_{12}, vitamin B_{12} (cyancobalamin); IF, intrinsic factor vitamin B_{12} binding protein; $KRPO_4$, Krebs-Ringer phosphate; $KRPO_4 - Ca^{++}/Mg^{++}$, $KRPO_4$ lacking $CaCl_2$ and $MgSO_4$; $KRPO_4 - Ca^{++}/Mg^{++}$ + EDTA, $KRPO_4 - Ca^{++}/Mg^{++}$ containing 0.001 M Na_2EDTA; NIF, gastric nonintrinsic factor B_{12} binding protein.

Schilling tests bind to ileal IF-B_{12} binding sites when complexed with B_{12} (17). Binding studies using crude gastric preparations are difficult to interpret because of the presence of B_{12} binding proteins that lack in vivo IF activity as well as the possible presence of additional gastric inhibitors or stimulators. Uncertainty also remains, therefore, with regard to (*d*) the affinity of the IF-B_{12} complex for the ileal B_{12} binding site; (*e*) the number of such binding sites present in ileal mucosa; and (*f*) the relative ability of IF obtained from different species to facilitate ileal B_{12} binding (1, 2, 18, 19).

Because of the ambiguities enumerated above, we have studied IF-facilitated B_{12}-binding utilizing homogeneous preparations of human IF, hog IF, hog gastric non-IF B_{12} binding protein (hog NIF), human plasma transcobalamin II, and B_{12} binding proteins isolated from human saliva, milk, and granulocytes. This report is concerned with the results of these studies.

METHODS

Preparation of intestinal mucosal homogenates. Guinea pigs, rabbits, hamsters, mice, and rats were decapitated. The intestine from pylorus to cecum was removed and placed on ice, and the lumen was rinsed with ice-cold isotonic saline. The intestine was divided in half, and the mucosa was scraped from the noneverted segments with glass microscope slides in a manner similar to that described by Sullivan, Herbert, and Castle (7). Hog, monkey, dog, and bovine small intestines were obtained within 45 min of death and placed on ice. Appropriate segments were opened along the mesenteric border and rinsed gently in ice-cold isotonic saline, and the mucosa was scraped free with glass microscope slides. Segments of human jejunum and distal ileum were obtained from patients undergoing ileojejunal bypass for morbid obesity and placed on ice. These segments were processed, and the mucosa was scraped free as described above for the hog and other large animals.

Mucosal scrapings from individual intestinal segments of large animals, or from pooled corresponding segments of small animals, were weighed and suspended in 10 vol (vol/wt) of ice-cold 0.14 M NaCl, 0.005 M KCl, 0.0025 M $CaCl_2$, 0.00125 M $MgSO_4$, 0.005 M potassium phosphate pH 7.4 (Krebs-Ringer phosphate, $KRPO_4$). Each suspension was homogenized in a Waring blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 30 s, divided into 10-ml aliquots, and stored at $-20°C$.

Homogenates were thawed immediately before use and were rehomogenized at 4°C with approximately 10 strokes of a motor-driven Teflon pestle in a fitted glass tube. Homogenates were centrifuged at 10,000*g* at 4°C, and the pellets were suspended with a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.) in $KRPO_4$ lacking $CaCl_2$ and $MgSO_4$ ($KRPO_4 - Ca^{++}/Mg^{++}$) and recentrifuged. The pellet was washed twice more, and sufficient $KRPO_4 - Ca^{++}/Mg^{++}$ was added to the final pellet to make the total value equal to 10 ml.

Assay of B_{12}. [^{57}Co]B_{12} (Amersham-Searle Corp., Arlington Heights, Ill., 150–200 μCi/μg) was diluted with nonradioactive crystalline B_{12} (Sigma Chemical Corp., St. Louis, Mo.) to achieve specific activities of 20–40 μCi/μg. Items containing [^{57}Co]B_{12} were assayed by measuring radioactivity in a Packard γ scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Solutions of crystalline B_{12} dissolved in water were assayed by measuring the absorbance at 361 nm and 550 nm. Molar extinction coefficients of $E_{1\,cm}$ 361 = 27,700 and $E_{1\,cm}$ 550 = 8,680 were used (20). The values for B_{12} concentration obtained at each wave length always agreed within 5%, and the average value was used. The endogenous B_{12} content of ileal homogenates was assayed by the isotope dilution technique of Lau, Gottlieb, Wasserman, and Herbert (21).

Assay of B_{12}-binding activity. B_{12}-binding activity was assayed by a modification (22) of the charcoal adsorption technique of Gottlieb, Lau, Wasserman, and Herbert (23).

Gel filtration. Preparation and calibration of columns of Sephadex G-150, fine grade, were performed as described previously (22).

Preparation of B_{12}-binding proteins. Human IF (22), hog IF (24), hog NIF (24), human plasma transcobalamin II (25), human granulocyte B_{12}-binding protein (26), human milk B_{12}-binding protein,[2] and human salivary B_{12}-binding protein[2] were purified as previously described. All preparations were homogeneous when measured by polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. Greater than 98% of the B_{12}-binding activity present in the preparations of human IF and hog IF employed was inhibited by anti-IF antibody obtained from the serum of a patient with pernicious anemia (22). The preparations of human IF and hog IF employed also were active in vivo based on Schilling tests (22, 24).

Saturation of B_{12}-binding proteins with [^{57}Co]B_{12}. A threefold excess (based on B_{12}-binding activity) of [^{57}Co]-B_{12} (20–40 μCi/μg B_{12}) was added to individual B_{12}-binding proteins (1–3 μg protein/ml) in 7.5 M guanidine-HCl containing 0.1 M potassium phosphate, pH 7.5. Proteins were dialyzed subsequently for 72 h at 4°C against 2,000 vol of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl, with dialysate changes at 24 and 48 h. Greater than 99% of unbound B_{12} was removed under these conditions. Protein preparations devoid of B_{12} were prepared in the same manner except that B_{12} was not added before dialysis. Dialyzed protein preparations were stored at $-20°C$. B_{12}-binding proteins were diluted in $KRPO_4 - Ca^{++}/Mg^{++}$ before being used for intestinal mucosal binding studies.

Assay of B_{12}-binding to intestinal mucosal homogenates. Incubations were performed in 10-mm × 75-mm glass test tubes presoaked for 2 h before use in a solution of bovine serum albumin, 1 mg/ml in distilled water. The tubes were aspirated to dryness subsequently with a vacuum aspirator. Millipore filters (Millipore Corp., Bedford, Mass.) (1.2 μm mean pore size-RA 02500) were presoaked in the same bovine serum albumin solution for 4 h before use.

Standard incubation mixtures contained the following components in order of addition: first, 0.72 ml of $KRPO_4$; second, 0.2 ml of intestinal mucosal homogenate suspended in $KRPO_4 - Ca^{++}/Mg^{++}$; and third, 0.08 ml of the solution containing free [^{57}Co]B_{12} or [^{57}Co]-B_{12} bound to protein. This solution contained three parts 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl, and eight parts $KRPO_4 - Ca^{++}/Mg^{++}$. Incubation mixtures were prepared at 4°C and placed in a 22°C water bath for 5 min before the addition of [^{57}Co]B_{12}. After standing at 22°C for an additional 180–210 min, the intestinal mucosal homogenates were col-

[2] Burger, R. L., and R. H. Allen. Manuscript in preparation.

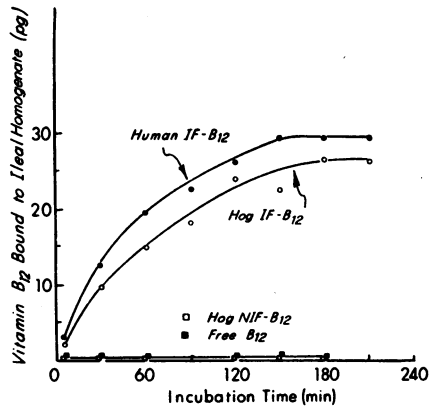

FIGURE 1 Time course of EDTA-inhibitable B_{12} binding to guinea pig ileal mucosal homogenate. The concentration of B_{12} in the standard incubation medium was 100 pg/ml. ■, free B_{12}; □, hog NIF-B_{12}; ○, hog IF-B_{12}; ●, human IF-B_{12}.

lected on Millipore filters by vacuum filtration. Assay tubes and filters were rinsed three times with 4 ml of incubation solution, and the filters were assayed directly for $[^{57}Co]B_{12}$ in a Packard γ scintillation counter. Assays were performed in duplicate, and the average value was used. Duplicates varied by less than 10%. In most experiments, duplicate assays were also performed in which $KRPO_4$ was replaced with $KRPO_4 - Ca^{++}/Mg^{++} + 0.001$ M Na_2 EDTA ($KRPO_4 - Ca^{++}/Mg^{++} +$ EDTA). The difference between B_{12} bound to intestinal mucosal homogenates in $KRPO_4$ and $KPRO_4 - Ca^{++}/Mg^{++} +$ EDTA was termed the "EDTA-inhibitable" fraction.

Determination of binding constants. The association constant, K_a for the binding of IF-B_{12} to ileal mucosal homogenate binding sites is defined as

$$K_a = \frac{[\text{IF-B}_{12}]_{\text{bound}}}{[\text{ileal IF-B}_{12}\text{ binding site}]_{\text{free}}\,[\text{IF-B}_{12}]_{\text{free}}},$$

where $[\text{IF-B}_{12}]_{\text{bound}}$ is the concentration of IF-B_{12} bound to ileal IF-B_{12}-binding sites, $[\text{ileal IF-B}_{12}\text{-binding site}]_{\text{free}}$ is the concentration of ileal IF-B_{12}-binding sites unoccupied by IF-B_{12}, and where $[\text{IF-B}_{12}]_{\text{free}}$ is the concentration of IF-B_{12} free in solution. Defined in this way, the total number of ileal IF-B_{12}-binding sites is equal to $[\text{IF-B}_{12}]_{\text{bound}} +$ $[\text{ileal IF-B}_{12}\text{-binding sites}]_{\text{free}}$. Data from ileal mucosal homogenate binding studies, performed with varying amounts of human IF-B_{12} and hog IF-B_{12}, were obtained, and the method of Steck and Wallach (27) was used to calculate the value for K_a and for the total number of ileal mucosal homogenate binding sites.

Precipitation of IF-B_{12} with anti-IF antibody. Sera from pernicious anemia patients were assayed for binding antibodies to human IF-B_{12} by a modification of the method of Rothenberg and Huhti (15). Test tubes containing 0.5 ml of serum consisting of varying amounts of normal and antibody-positive sera and 1.0 ml of 0.075 M potassium phosphate, pH 7.5, 0.375 M NaCl, and 100 pg of $[^{57}Co]B_{12}$ bound to human IF were incubated at 22°C for 30 min. The tubes were then placed in an ice bath and 1.5 ml of cold 30% Na_2SO_4 was added. After standing for an additional 10 min, the tubes were centrifuged at 20,000g for 10 min and 1 ml of supernatant solution was removed and assayed for $[^{57}Co]B_{12}$.

RESULTS

General properties of ileal IF-B_{12} binding. The properties of homogeneous human and hog IF-B_{12} binding to guinea pig, human, and hog ileal mucosal homogenates are illustrated in Table I. Under standard conditions at a B_{12} concentration of 100 pg/ml, 13.6%–23.0% of human IF-B_{12} and 16.1%–22.7% of hog IF-B_{12} were bound to these ileal mucosal homogenates. When the standard incubation medium contained 0.001 M Na_2-EDTA, there was no effect on binding. When Ca^{++} and Mg^{++} were omitted from the incubation medium, there was no effect

TABLE I

Characterization of IF-B_{12} Binding to Ileal Mucosal Homogenates

Assay conditions	$[^{57}Co]B_{12}$ bound to ileal mucosal homogenates					
	Human IF-B_{12}			Hog IF-B_{12}		
	guinea pig*	human*	hog*	guinea pig*	human*	hog*
	pg	*pg*	*pg*	*pg*	*pg*	*pg*
Standard‡	23.0	16.6	13.6	22.7	16.1	17.4
+0.001 M Na_2 EDTA	23.1	16.5	13.4	22.9	16.2	17.1
$-Ca^{++}$, $-Mg^{++}$	17.1	16.5	13.1	16.7	15.8	17.3
$-Ca^{++}$, $-Mg^{++}$, +0.001 M Na_2 EDTA	0.4	0.5	1.3	0.8	1.3	1.3
+Normal serum§	22.8	16.8	13.7	22.4	16.3	17.0
+Anti-IF antibody serum§	1.0	1.6	0.8	0.9	1.4	1.2
−Ileal homogenate	0.2	0.2	0.2	0.2	0.2	0.2
−Ileal homogenate +Jejunal homogenate	0.4	0.7	1.7	0.8	0.9	1.3

* Homogenate species.

‡ Assays contained 0.72 ml of $KRPO_4$; 0.08 ml containing 100 pg of $[^{57}Co]B_{12}$ in a solution consisting of eight parts $KRPO_4 - Ca^{++}/Mg^{++}$ and three parts 0.05 M potassium phosphate, 0.75 M NaCl; and 0.2 ml of ileal mucosal homogenate.

§ 0.2 ml of serum was added in place 0.2 ml of $KRPO_4$.

on human IF-B_{12} and hog IF-B_{12} binding to human and hog ileal mucosal homogenates and only a slight but reproducible inhibition of binding to guinea pig ileal mucosal homogenate. When 0.001 M Na_2-EDTA was present in Ca^{++}- and Mg^{++}-free incubation medium, there was greater than 90% inhibition of human IF-B_{12} and hog IF-B_{12} binding to the ileal mucosal homogenates of all three species. Marked inhibition of IF-B_{12} binding to ileal mucosal homogenates was also observed in the presence of serum containing antibodies to the IF-B_{12} complex, and when the ileal mucosal homogenate was either omitted from the incubation system or replaced with jejunal mucosal homogenate. Similar small amounts of IF-B_{12} binding were observed when these last experiments were performed in the presence of 0.001 M Na_2-EDTA in standard medium lacking added Ca^{++} and Mg^{++} (data not shown).

Protein specificity. The results of experiments performed to determine the ileal mucosal homogenate binding of 100 pg/ml B_{12}, present in either free form or bound to each of seven different homogeneous B_{12}-binding proteins, are presented in Table II. Under standard incubation conditions, B_{12} bound to human IF and hog IF was bound to ileal mucosal homogenates in significantly greater amounts than was free B_{12} or B_{12} bound to hog NIF, human transcobalamin II, or human milk, salivary, and granulocyte B_{12}-binding proteins. It is also important to note that ileal mucosal homogenate B_{12} binding was inhibited significantly by 0.001 M EDTA in the absence of added Ca^{++} and Mg^{++} only in the cases of human IF-B_{12} and hog IF-B_{12}.

Time-course of ileal IF-B_{12} binding. The time-course of EDTA-inhibitable B_{12} binding to guinea pig ileal mucosal homogenates is presented in Fig. 1. B_{12} binding

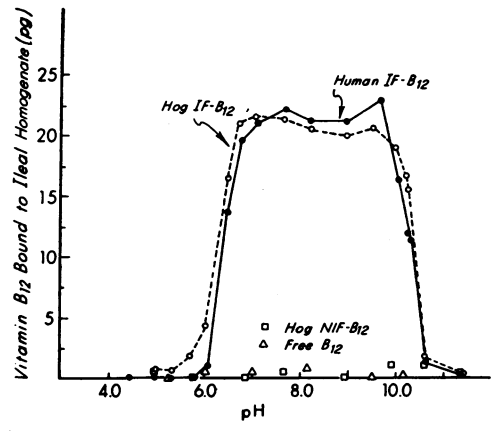

FIGURE 2 Effect of pH on EDTA-inhibitable B_{12} binding to guinea pig ileal mucosal homogenate. Potassium phosphate was omitted from the standard incubation medium, which was adjusted to contain 0.015 N acetic acid, 0.015 M tricine, 0.015 M glycine, and sufficient NaOH to achieve the desired pH value. These changes were required to avoid precipitates of calcium and phosphate at pH values above 8 as well as to obtain sufficient buffer capacity from pH 4 to pH 11. The B_{12} concentration in the incubation medium was 100 pg/ml. The incubation period was 180 min. △, free B_{12}; □, hog NIF-B_{12}; ○, hog IF-B_{12}; ●, human IF-B_{12}.

reaches a maximum with human IF-B_{12} and hog IF-B_{12} at 150–210 min. Similar rates of binding were observed with ileal mucosal homogenates of all other species (data not shown). These findings led to our choice of 180–210 min incubations for the standard assay. Only negligible EDTA-inhibitable binding of free B_{12} or hog NIF-B_{12} occurs over the entire incubation period.

pH dependence of ileal IF-B_{12} binding. The pH dependence of EDTA-inhibitable B_{12} binding to guinea pig

TABLE II

Effect of Homogenous B_{12} Binding Proteins on B_{12} Binding to Ileal Mucosal Homogenates

	[^{57}Co]B_{12} bound to ileal mucosal homogenates								
	Guinea pig homogenate			Human homogenate			Hog homogenate		
B_{12} binding protein present	(1) standard*	(2) $-Ca^{++}$, $-Mg^{++}$ +0.001 M Na_2 EDTA*	(1)–(2) EDTA-inhibitable	(1) standard*	(2) $-Ca^{++}$, $-Mg^{++}$ +0.001 M Na_2 EDTA*	(1)–(2) EDTA-inhibitable	(1) standard*	(2) $-Ca^{++}$, $-Mg^{++}$ +0.001 M Na_2 EDTA*	(1)–(2) EDTA-inhibitable
	pg	pg	pg	pg	pg	pg	pg	pg	pg
None	2.4	2.1	0.3	1.6	1.5	0.1	8.6	8.2	0.4
Human IF	23.1	0.4	22.7	16.6	0.5	16.1	13.6	1.0	12.6
Hog IF	22.7	0.8	21.9	16.1	1.3	14.8	17.4	1.3	16.1
Hog NIF	1.5	1.0	0.5	2.4	2.5	0.1	3.6	3.2	0.4
Human transcobalamin II	1.8	2.6	−0.8	1.4	1.6	−0.2	8.6	7.5	1.1
Human milk B_{12} binder	0.0	0.0	−0.0	0.3	0.0	0.3	0.2	0.0	0.2
Human salivary B_{12} binder	0.2	0.1	0.1	0.5	0.5	0.0	0.1	0.4	−0.3
Human granulocyte B_{12} binder	0.6	0.4	0.2	0.5	0.4	0.1	1.1	0.8	0.3

All assays were performed at a concentration of [^{57}Co]B_{12} of 100 pg/ml.
* Assay medium.

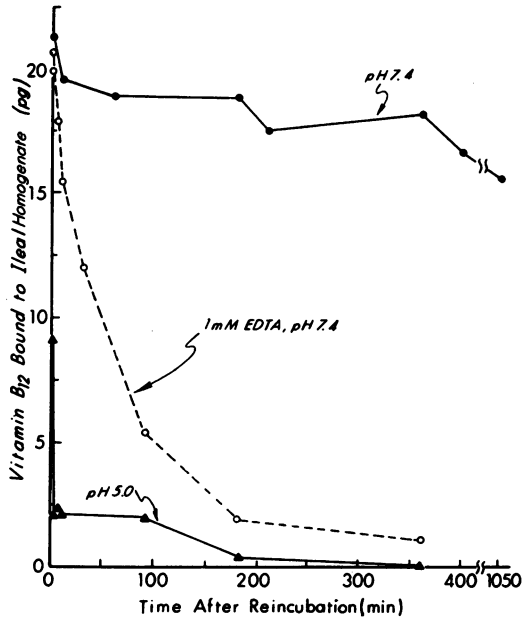

FIGURE 3 Time course of human IF-B_{12} release from guinea pig ileal mucosal homogenate. Human IF-B_{12} (100 pg [^{57}Co]-B_{12}/ml) was incubated for 180 min at 22°C in 1.0 ml of standard ($KRPO_4$) incubation medium containing guinea pig ileal mucosal homogenate. Individual assay tubes were centrifuged subsequently and the pellets were washed three times in standard incubation medium. The final pellets were incubated in 4.0 ml of reincubation medium for various time periods at 22°C, collected on Millipore filters, and assayed for [^{57}Co]B_{12}. The reincubation media consisted of ●, $KRPO_4$ (pH 7.4); ○, $KRPO_4 - Ca^{++}/Mg^{++} + EDTA$ (pH 7.4); and △, $KRPO_4 - Ca^{++}/Mg^{++}$ containing 0.001 N citric acid and sufficient HCl to adjust the pH to 5.0. Based on the k_a (13.0×10^9 M^{-1}) for IF-B_{12} and this ileal homogenate preparation, and on the number of ileal binding sites (1.2×10^{12} g wet wt) determined for this homogenate preparation (see below), the expected amount of IF-B_{12} bound to ileal binding sites at equilibrium after reincubation in standard medium was calculated to be 2.0 pg.

ileal mucosal homogenates is demonstrated in Fig. 2. Below pH 5.6 and above pH 10.5 IF-mediated B_{12} binding is minimal, and negligible free B_{12} or hog NIF-B_{12} binding occurs from pH 5.0 to pH 11.5. Optimal IF-mediated B_{12} binding occurs between pH 6.5 and pH 9.5. Similar curves were observed with hog and human ileal mucosal homogenates.

Saturability and specificity of ileal IF-B_{12} binding. To study the saturability and specificity of EDTA-inhibitable IF-B_{12} binding to ileal mucosal homogenates, 100-fold excesses of nonradioactive human IF-B_{12}, hog IF-B_{12}, hog NIF-B_{12}, free B_{12}, and human IF, hog IF, and hog NIF devoid of B_{12} were added to standard assay mixtures containing 100 pg of [^{57}Co]B_{12} bound to human or hog IF. The results are presented in Table III and demonstrate that a limited number of binding sites for human IF-B_{12} and hog IF-B_{12} exists, because 100-fold excesses of nonradioactive human and hog IF-B_{12} cause greater than 90% inhibition of IF-[^{57}Co]B_{12} binding to ileal mucosal homogenates. The finding that nonradioactive human IF-B_{12} inhibits hog IF-[^{57}Co]B_{12} binding to ileal mucosal homogenates, and that nonradioactive hog IF-B_{12} inhibits human IF-[^{57}Co]B_{12} binding to ileal mucosal homogenates also suggests that human IF-B_{12} and hog IF-B_{12} bind to the same ileal mucosal homogenate binding sites. The finding that 100-fold excesses of nonradioactive free B_{12} and hog NIF-B_{12} cause no detectable inhibition of either human IF-[^{57}Co]B_{12} or hog IF-[^{57}CO]B_{12} ileal mucosal homogenate binding indicates that free B_{12} and hog NIF-B_{12} have affinities for ileal mucosal IF-B_{12}-binding sites that are at least three orders of magnitude lower than that of human IF-B_{12} and hog IF-B_{12}. Data in Table III also support similar conclusions about the maximal possible affinities of human IF and hog IF devoid of B_{12} for human and hog ileal mucosal homogenate IF-B_{12}-binding sites. A reproducible 20% inhibition of human IF-[^{57}Co]B_{12} and hog IF-[^{57}Co]B_{12} binding to guinea pig ileal mucosal homogenataes was effected by 100-fold excesses of human and hog IF devoid of B_{12}, suggesting that they may have definite affinities for guinea pig ileal mucosal homogenate IF-B_{12}-binding sites, although such affinities appear to be approximately $2\frac{1}{2}$ orders of magnitude lower than those of human IF-B_{12} and hog IF-B_{12}. This degree of inhibition is also compatible, however, with the presence of small amounts, i.e. approximately 25 pg, of free endogenous B_{12} in the guinea pig homogenate. This possibility is supported by the fact that 0.2 ml of washed guinea pig ileal mucosal homogenate contains approximately 500 pg of endogeneous B_{12}. Human and hog ileal mucosal homogenates contained 50 and 30 pg B_{12}/0.200 ml, respectively. The amount of this endogenous B_{12} that becomes free during the incubation period has not been determined.

Release of IF-B_{12} from ileal mucosal homogenate. The results of experiments performed to determine the rate of release of human IF-B_{12} bound to guinea pig ileal mucosal homogenate are presented in Fig. 3 and indicate that IF-B_{12} is released slowly under standard assay conditions with a $t_{\frac{1}{2}}$ of release of greater than 18 h. Fig. 3 also demonstrates that release is faster in a medium devoid of Ca^{++} and Mg^{++} and containing EDTA ($t_{\frac{1}{2}}$ = 40–60 min) and even more rapid at pH 5.0 ($t_{\frac{1}{2}}$ = 5–10 min). Similar results were obtained when the release of hog IF-B_{12} was studied.

Reversibility of ileal IF-B_{12} binding. The results of a large-scale experiment performed to obtain B_{12} released from ileal mucosal homogenate are presented in Table IV. When the B_{12} released from the guinea pig ileal mucosal homogenate at pH 5.0 with EDTA was dialyzed at

TABLE III

Specificity and Saturability of EDTA-Inhibitable IF-B_{12} Binding to Ileal Mucosal Homogenates

Nonradioactive item present in 100-fold excess	[^{57}Co]B_{12} bound to ileal mucosal homogenates					
	Human IF-B_{12}			Hog IF-B_{12}		
	guinea pig*	human*	hog*	guinea pig*	human*	hog*
	pg	*pg*	*pg*	*pg*	*pg*	*pg*
None	13.8	12.0	12.5	13.4	12.9	10.0
Human IF + B_{12}	0.7	0.6	0.0	0.6	0.4	0.4
Hog IF + B_{12}	0.3	0.2	0.4	0.8	0.6	0.9
Hog NIF + B_{12}	13.5	11.8	12.3	13.1	12.7	10.3
Free B_{12}	14.3	12.0	12.0	14.4	12.7	10.0
Human IF	12.5	12.9	12.6	11.2	14.1	10.2
Hog IF	11.4	12.3	11.3	10.1	12.4	10.0

* Homogenate species.

Assays were performed at a concentration of [^{57}Co]B_{12} of 100 pg/ml. Standard assay conditions were employed as described in Methods except that ileal mucosal homogenate was the last item added to incubation mixtures.

4°C for 24 h against 40 vol of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl, less than 10% of the B_{12} was recovered in the dialysate, according to measurements of radioactivity. This observation suggested that the B_{12} released from ileal mucosal homogenate was bound to a macromolecule. This was confirmed by the observation that the dialyzed, released B_{12} eluted from Sephadex G-150 with an apparent molecular weight of 65,000, the same as the apparent molecular weight of human IF-B_{12} under these conditions (*22*). The released and dialyzed B_{12} was bound to guinea pig ileal homogenate in an amount comparable to that of human IF-B_{12}, and this observation, together with the finding that released B_{12} is precipitated with anti-IF antibody in 15% Na_2SO_4 in a manner equivalent to human IF-B_{12}, demonstrates that the human IF-B_{12} complex is bound to guinea pig ileal mucosal homogenate under our standard assay conditions and that this binding is reversible.

Binding constants and species specificity of ileal IF-B_{12} binding. The amounts of EDTA-inhibitable IF-B_{12} binding to ileal mucosal homogenates at varying concentrations of IF-B_{12} were used to calculate association constants for human and hog IF-B_{12} and guinea pig, human, and hog ileal mucosal homogenates as presented in Fig. 4. The concentration of IF-B_{12} bound to the ileal mucosal homogenates, $[\text{IF-}B_{12}]_{bound}$, was calculated under the assumption that each ileal mucosal homogenate binding site binds one molecule of IF-B_{12}. The concentration of IF-B_{12} remaining free in solution, $[\text{IF-}B_{12}]_{free}$, was calculated by subtracting $[\text{IF-}B_{12}]_{bound}$ from the total concentration of IF-B_{12} present in the incubation mixture. We have demonstrated (*22, 24*) that human IF and hog IF both contain single B_{12}-binding sites, but it is important to note that our calculation of $[\text{IF-}B_{12}]_{free}$ does assume that under our standard assay conditions IF-B_{12} does not aggregate to form a series of oligomers, as can occur (*22, 24*). This assumption is supported by the finding that when human IF-B_{12} (100 pg B_{12}/ml) was incubated at 22°C for 180 min in standard assay medium and then placed on a 2.0 × 60-cm column of Sephadex G-150 equilibrated at 22°C with standard assay medium, more than 90% of IF-B_{12} eluted with an apparent molecular weight of 65,000, i.e. the apparent monomeric molecu-

TABLE IV

Release of [^{57}Co]B_{12} from Guinea Pig Ileal Mucosal Homogenate Incubated Previously with Human IF-[^{57}Co]B_{12}

Item	Volume	[^{57}Co]B_{12}	
	ml	*pg*	%
Incubation mixture in $KRPO_4$	200	20,000	100.0
Supernate after centrifugation			
1. Initial	190	11,700	58.5
2. 1st wash in $KRPO_4$	200	660	3.3
3. 2nd wash in $KRPO_4$	200	360	1.8
4. 3rd wash in $KRPO_4$	200	330	1.6
5. After suspension in pH 5.0 $KRPO_4$ − Ca^{++}/Mg^{++} + EDTA containing 0.001 N citric acid	27.5	5,900	29.5
Final pellet		460	2.3
Supernate 5 after dialysis at 4°C against 1.0 liter, of 0.05 M potassium phosphate pH 7.5, 0.75 M NaCl	26.0	5,510	27.6

A standard incubation mixture was prepared in 200 ml of $KRPO_4$ medium containing 40 ml of guinea pig ileal mucosal homogenate and 20000 pg of [^{57}Co]B_{12} bound to human IF. After an incubation period of 180 min at 22°C, the mixture was centrifuged at 10,000*g* and the pellet washed three times in $KRPO_4$ medium and suspended for 15 min in pH 5.0 $KRPO_4$ − Ca^{++}/Mg^{++} + EDTA containing 0.001 N citric acid. The suspension was then recentrifuged.

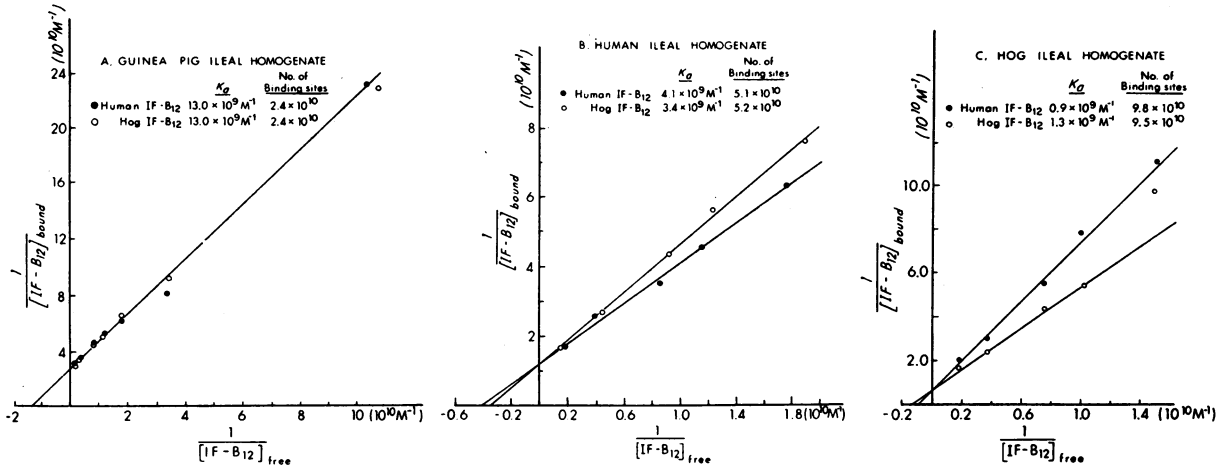

FIGURE 4 Double reciprocal plots of EDTA-inhibitable IF-B_{12} binding to ileal mucosal homogenates *versus* IF-B_{12} concentration. Values for K_a and the number of IF-B_{12} binding sites/0.2 ml of ileal mucosal homogenate were determined as described in Methods. A, guinea pig ileal mucosal homogenate; B, human ileal mucosal homogenate; C, hog ileal mucosal homogenate.

lar weight of human IF-B_{12} (*22*). A similar result was obtained when human IF-B_{12} was incubated and chromatographed on Sephadex G-150 in medium containing $KRPO_4 - Ca^{++}/Mg^{++} + EDTA$.

EDTA-inhibitable binding of human IF-B_{12} and hog IF-B_{12} to intestinal mucosal homogenates prepared from rabbits, hamsters, dogs, mice, and monkeys was also observed. The specificity and saturability of ileal IF-B_{12} binding in each species was demonstrated by the findings that (*a*) EDTA-inhibitable IF-B_{12} binding was limited to ileal mucosal homogenates and was not observed with jejunal mucosal homogenates; (*b*) more than 90% of EDTA-inhibitable IF-B_{12} binding was inhibited by a 100-fold excess of nonradioactive IF-B_{12}; (*c*) more than 90% of EDTA inhibitable IF-B_{12} binding was inhibited with anti-IF antibody; (*d*) no EDTA-inhibitable binding of free B_{12} or hog NIF-B_{12} was observed; and (*e*) plots of $1/[\text{IF-B}_{12}]_{bound}$ vs $1/[\text{IF-B}_{12}]_{free}$ were linear. EDTA-inhibitable IF-B_{12} binding was not observed in proximal, mid, or distal mucosal homogenates from rat or bovine small intestine.

TABLE V
Species Specificity of EDTA-Inhibitable IF-B_{12} Binding to Ileal Mucosal Homogenates

Ileal homogenate	K_a		Number of binding sites per wet weight of ileal mucosa	
	Human IF-B_{12}	Hog IF-B_{12}	Human IF-B_{12}	Hog IF-B_{12}
Species	$10^9\ M^{-1}$	$10^9\ M^{-1}$	$10^{12}/g$	$10^{12}/g$
Guinea pig	13.0	13.0	1.2	1.2
Guinea pig	9.5	9.3	0.9	0.9
Rabbit	3.4	3.1	0.4	0.5
Human	4.1	3.4	2.5	2.6
Human	3.4	2.7	0.4	0.5
Human	5.6	5.0	0.4	0.4
Monkey	5.9	5.0	0.4	0.4
Dog	6.6	4.3	4.8	4.7
Hog	0.9	1.3	4.7	4.9
Hamster	0.4	1.1	1.8	1.6
Mouse	0.3	1.1	0.3	0.3
Rat	<0.1	<0.1	—	—
Bovine	<0.1	<0.1	—	—

Each set of determinations was performed with a different preparation of ileal mucosal homogenate. Values for human IF-B_{12} and hog IF-B_{12} were obtained simultaneously with each ileal mucosal homogenate preparation.

The data obtained from all of the species tested are presented in Table V. Values for K_a for human and hog IF-B_{12}, and different ileal mucosal homogenate preparations from the same species differed by as much as 50%, but the relative differences observed for the K_a for human IF-B_{12} and hog IF-B_{12} varied by less than 10%. Human IF-B_{12} appears to have a slightly greater affinity for human, monkey, and dog ileal mucosal IF-B_{12}-binding sites than does hog IF-B_{12}. The reverse appears true in the case of hog, and especially hamster and mouse ileal IF-B_{12}-binding sites. No significant differences between the number of human IF-B_{12}-binding sites and the number of hog IF-B_{12}-binding sites were observed in any individual ileal mucosal homogenate preparation, a finding consistent with our observation (see above) that human IF-B_{12} and hog IF-B_{12} bind to the same binding sites.

DISCUSSION

B_{12} bound to human and hog IF is bound to ileal mucosal homogenates in a number of different species in significantly greater amounts than is free B_{12}, or B_{12} bound to

hog NIF, human transcobalamin II, or B_{12}-binding proteins isolated from human milk, saliva, and granulocytes. Since these observations were made with homogeneous protein preparations, the observed differences in ileal B_{12} binding are due to structural differences in the individual B_{12}-binding proteins rather than to the presence of inhibitors or stimulators of ileal B_{12} binding that might exist in the crude tissue extracts or body fluids in which these proteins are found. The physiologic significance of the relatively small amounts of B_{12} binding to ileal mucosal homogenates observed with free B_{12} and with B_{12} bound to the B_{12}-binding proteins other than IF is not known. This type of binding appears to be different from IF-B_{12} ileal binding, however, in that it is not inhibited by EDTA, low pH, or anti-IF antibody and is not localized to the distal portion of the small intestine.

Our studies also indicate that gastric factors other than IF are not required during the actual process of IF-B_{12} binding to ileal binding sites, since homogeneous human and hog IF are able to facilitate B_{12} binding to ileal mucosal homogenates of the same order of magnitude that other investigators have observed with whole gastric juice in similar assays (4, 7–10). Gastric factors may exist that alter the IF molecule after its synthesis in the gastric mucosa, but if such factors do exist, their action must have occurred before our isolation of human and hog IF.

In previous studies, in which crude gastric juice was employed as the source of IF, some (2, 7, 9, 11, 14, 15) but not all (6, 8, 16) investigators have observed decreases in ileal B_{12} binding when increasing amounts of gastric juice, containing unsaturated B_{12}-binding activity, were added to their assay systems. The former observations have suggested that IF devoid of B_{12} might have an appreciable affinity for the ileal IF-B_{12}-binding site and thus be the cause of the inhibition of ileal IF-B_{12} binding. Other interpretations are available, however, and include the possibility that crude gastric juice contains substances other than IF that inhibit ileal IF-B_{12} binding. This possibility is suggested by the fact that Donaldson, Mackenzie, and Trier (11) observed that partially purified hamster IF-B_{12} was bound to hamster ileal brush borders in significantly greater amount than was unpurified hamster IF-B_{12}. We have not examined IF from all of the species utilized in the studies mentioned above, but our studies employing homogeneous human and hog IF indicate that these two proteins, devoid of B_{12}, have relatively low, if any, affinity for guinea pig, human, and hog ileal IF-B_{12}-binding sites. This suggests that the ileal IF-B_{12}-binding site either interacts with portions of both the B_{12} and IF molecules or that B_{12} binding to IF results in important conformational changes in that portion of the B_{12} and/or IF molecule that interacts with the ileal binding site. It is possible that the structures of human and hog IF are altered during their purification and that such alterations are responsible for their failure to bind significantly to ileal IF-B_{12}-binding sites in the absence of B_{12}. This type of alteration could have occurred when the proteins were exposed to high concentrations of guanidine-HCl during their purification but appears unlikely since human IF and hog IF can be renatured from 7.5 M guanidine-HCl in the absence of B_{12} with full preservation of their abilities to bind B_{12}, and facilitate B_{12} absorption as judged by Schilling tests (22, 24).

Human gastric juice and crude preparations of hog gastric mucosa contain B_{12}-binding proteins that lack IF activity, as judged by Schilling tests (17, 24, 28, 29). These proteins have been referred to as human gastric R binder and hog NIF. The source of human gastric R binder is not entirely clear, but recent studies (22) suggest that most, if not all, of this B_{12} binding protein may result from the contamination of gastric juice with saliva. We have recently isolated the human salivary B_{12}-binding protein [2] and hog NIF (24), and have shown that they differ from human and hog IF immunologically as well as in terms of molecular weight and amino acid and carbohydrate composition. The studies presented here indicate that neither the salivary protein nor hog NIF are able to facilitate B_{12} binding to ileal IF-B_{12}-binding sites. This observation, together with the fact that these proteins are present in gastric preparations in variable amounts relative to IF (7, 23, 30), may explain some of the conflicting data in the literature about the relative ability of human IF and hog IF to facilitate ileal B_{12} binding (1, 2, 10, 18, 31) and absorption (18, 19) in various species.

Human IF and hog IF both facilitate B_{12} binding to human, dog, monkey, hog, hamster, mouse, guinea pig, and rabbit intestine mucosal homogenates with affinities that range from 0.3×10^9 M^{-1} to 13×10^9 M^{-1}. Both IF-B_{12} complexes have similar affinities for guinea pig and rabbit ileal IF-B_{12}-binding sites, but differences in affinity appear to exist with respect to the other six ileal mucosal homogenates. These differences indicate that human IF and hog IF have different structures. This observation is consistent with our recent experiments (22, 24) demonstrating that human IF and hog IF do differ slightly, but significantly, in amino acid and carbohydrate composition, molecular weight, and in their interaction with anti-IF antibody and pseudo-B_{12} (α-adenyl cobamide cyanide).

Uncertainty exists about the fate of the IF-B_{12} complex after its attachment to the ileal mucosal IF-B_{12} binding site, but at some point B_{12} must be dissociated from IF since IF does not appear to enter the portal circulation with B_{12} (32, 33). The factor, or factors, responsible for this dissociation are undefined, but it is

possible that species specificity exists in this process and that this specificity may well differ from that involved in IF-B_{12} binding to ileal IF-B_{12}-binding sites. Because of this possibility, it is important to note that the demonstration that human IF-B_{12} binds to a particular species of ileal IF-B_{12}-binding site does not demonstrate that human IF-B_{12} is capable of facilitating actual B_{12} absorption in that species.

One additional point of caution about the interpretation of our results is that little is known about the fate of the IF-B_{12} complex during its passage from the stomach to the distal small intestine. During the passage the IF-B_{12} complex is exposed to a large number of gastric, pancreatic, and intestinal proteolytic enzymes, glycosidases, and other factors that could alter the structure of the IF-B_{12} complex before it binds to the ileal IF-B_{12}-binding site. This consideration is important since it suggests that under physiological in vivo conditions, IF-B_{12} binding to ileal IF-B_{12}-binding sites might be different from that observed with homogeneous IF isolated from gastric juice and gastric mucosa. Other recent studies (34–36), demonstrating B_{12} malabsorption in humans and rats with pancreatic insufficiency and its correction with pancreatic extracts and highly purified preparations of trypsin, suggest that differences might exist between in vivo and in vitro ileal IF-B_{12} binding.

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