Intrinsic factor-mediated intestinal absorption of cobalamin in the dog

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MARCOULLIS, GEORGE, AND SHELDON P. ROTHENBERG. *Intrinsic factor-mediated intestinal absorption of cobalamin in the dog.* Am. J. Physiol. 241 (Gastrointest. Liver Physiol. 4): G294–G299, 1981.—The purpose of these studies was to determine whether gastric intrinsic factor and the ileal intrinsic factor receptor participate in the process of cobalamin absorption in the dog. Physicochemical analysis of gastrointestinal fluids and mucosal extracts obtained 3–5 h after cyano[^{57}Co]-cobalamin was fed to dogs demonstrated that *1*) all cyano-[^{57}Co]cobalamin became bound to proteins during intraluminal transport; and *2*) mucosal cyano[^{57}Co]cobalamin in the extract of the ileal mucosa was bound to intrinsic factor, to intrinsic factor coupled to receptor protein, and to proteins with properties similar to R protein and transcobalamin II. A significant fraction of the cyano[^{57}Co]cobalamin in the mucosal extract was membrane bound and, upon solubilization with Triton X-100, was found to contain immunoreactive intrinsic factor that, however, could no longer couple to the isolated receptor. The formation of the complex of cobalamin with intrinsic factor and the receptor protein and the selective accumulation of cobalamin in the ileum indicate that the intrinsic factor-mediated mechanism for absorption of this vitamin is active in the dog.

membrane receptor; nutrient transport; transmembrane transport; vitamin B_{12}; transcobalamins; alimentary tract

THE INTESTINAL ABSORPTION of cobalamin (Cbl) in most mammals is mediated by a complex process that includes the binding of the vitamin to intrinsic factor (IF) in the upper alimentary tract and transit of the IF·Cbl to the distal ileum (3). The IF moiety of the complex attaches to specific receptors on the microvillus membranes of the epithelial mucosa by a calcium- and pH-dependent interaction (8, 16). Although this IF-dependent mechanism of Cbl absorption has been observed with most mammals (12), there have been conflicting observations on the process by which Cbl is assimilated in the dog. One series of reports (37–39) appeared to demonstrate that this animal does not secrete IF and that Cbl absorption was by simple or facilitated diffusion. In addition, other studies (17) indicated that dog gastric Cbl-binding proteins do not enhance the vitamin absorption in humans.

These reports notwithstanding, the more recent studies of Hooper and co-workers (18) have demonstrated that dog ileal mucosa preparations can bind the heterologous human and hog IF·Cbl complexes, and more specific studies in this laboratory have demonstrated that the dog ileal mucosa contains a binding factor for IF that has a molecular radius of 12.9 nm and stoichiometrically binds IF·Cbl complexes. These same studies have also demonstrated the presence of IF in dog gastric juice and pyloric extracts by its molecular radius of 3.54 nm, by its reactivity with antiserum to human IF, by its reactivity with solubilized IF receptor, and by its lack of affinity for the binding of cyanocobinamide (29).

These biochemical and immunologic studies that have demonstrated that both IF and the IF receptor protein are present in the dog do not by themselves ensure that these proteins are actually involved in the ileal absorption of Cbl in the dog. As Hall (14) has suggested in the past, the critical study to answer similar questions would be an analysis of Cbl binding in the alimentary tract after intake of labeled Cbl. Accordingly, to elucidate the actual process of Cbl absorption in the canine species, dogs were fed physiological doses of Cbl, and the distribution of protein-bound forms of the vitamin were analyzed in the succus entericus and mucosa of the small intestine.

MATERIALS AND METHODS

All animal experimentation included in this report was conducted in conformity with the "Guiding Principles in the Care and Use of Animals," approved by the Council of the American Physiological Society.

Radioisotopes and protein preparations. Cyano-[^{57}Co]cobalamin (CN[^{57}Co]Cbl; sp act, 100–150 Ci/g) and $^{3}H_2O$ were purchased from Amersham/Searle. Purified human serum albumin (HSA; Kabis, Sweden), immunoglobulin G (IgG), and apoferritin (Schwartz/Mann) were labeled (13) with iodine-125 (^{125}I; Union Carbide) and used as molecular weight marker proteins for gel filtration chromatography.

Preparation of tissue extracts and fluids. While under pentobarbital sodium anesthesia (Nembutal, 30 mg/kg body wt), mongrel dogs weighing 20–25 kg were killed by lethal injection (0.3 ml/kg body wt) of Euthanasia solution T-61 (National Laboratories). Within a few minutes of death, the small intestine was removed and divided with ligatures into eight equal segments, each one measuring approximately 50 cm in length. The gastric and intestinal succus contents were collected separately, mixed with the corresponding washings (50 ml from each segment), depepsinized, clarified by centrifugation at 27,000 *g* for 30 min at 4°C, and stored at −20°C. The intestinal segments and the stomach were opened longitudinally and rinsed five times with Ringer-bicarbonate

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solution (pH 7.2) that was lacking calcium (approximately 500-ml buffer per segment).

The mucosae were then scraped on iced plates, suspended in 2 vol of buffer, and homogenized in the cold in a Waring blender for 1 min at 15,000 rpm. The homogenate from the pyloric mucosa was centrifuged at 27,000 *g* for 30 min, and the supernatant (designated as "pyloric extract") was depepsinized and stored at −20°C. The homogenates from the segments of the small intestine were further disrupted by mechanical shearing forces for 3 min in a Braun MSK fractionator using 0.10- to 0.11-mm glass microspheres. These homogenates were centrifuged at 27,000 *g* for 30 min and then at 100,000 *g* for 1 h, and the supernatants as well as the particulates were stored at 4°C. The extracts from the two most distal intestinal segments (measuring 1 m in length) will henceforth be referred to as "ileal extracts."

To study the distribution of Cbl in the alimentary tract of the dog during the natural process of absorption, 100–3,000 ng (10 μCi) of CN[^{57}Co]Cbl were instilled through a nasogastric tube into the lumen of five animals prefasted for 12 h. Approximately 3–5 h later, the dogs were killed, and the gastric and intestinal juices and tissue extracts were prepared as described above. In addition, for these in vivo experiments, the pellet obtained after the 100,000-*g* centrifugation of the disrupted mucosa was suspended in 4–5 vol of Ringer buffer and homogenized as described above. Each homogenization was followed by centrifugation at 100,000 *g*. This was repeated three times, the three supernatants were pooled, and the radioactivity was measured.

The final pellet was now suspended in 30 vol of 50 mM tris(hydroxymethyl)aminomethane hydrogen chloride (Tris-HCl) buffer (pH 7.5) containing 0.05% (vol/vol) Triton X-100, 154 mM NaCl, 0.02% (wt/vol) NaN_3, and 0.2 mM phenylmethylsulfonyl fluoride and homogenized in the cold, first in the Waring blender for 45 s at 15,000 rpm and second with the glass microspheres in the MSK homogenizer for 30 s. The final homogenate was kept on a slowly turning rotation mixer for 50 h at 4°C, ultrafiltered under negative pressure, and then centrifuged at 100,000 *g*. The final concentrated supernatant, obtained after the 100,000-*g* centrifugation, was analyzed by gel filtration as described below.

Chromatography. The complexes of CN[^{57}Co]Cbl with macromolecules were determined by filtration at 4°C through Sephadex G-200 and Sepharose 4B (Pharmacia, Sweden) using 2.5 x 100 cm chromatography columns equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.4) containing 154 mM NaCl, 1 mM Ca^{2+}, 0.02% (wt/vol) NaN_3, and 0.05% (vol/vol) Triton X-100. Fractions of 1.5–2.5 ml were collected at a constant flow rate of 12–13 ml/h. With each run, the appropriate standard marker proteins labeled with ^{125}I were simultaneously filtered to calibrate the columns for estimation of molecular size.

RESULTS

The means ± SD (n = 6) of the unsaturated Cbl-binding capacity (UCblBC) for extracts of the pyloric and ileal mucosa were 2,420 ± 380 and 85 ± 22 ng/g wet wt, respectively. Gel filtration chromatography through Sephadex G-200 after incubation of the pyloric extracts and gastric juice separately with cyanocobinamide and anti-IF serum (29) demonstrated that 10% of the total UCblBC was IF and the remainder was R protein.

The average distribution of radioactivity in the gastrointestinal tract 3–5 h after oral administration of CN[^{57}Co]Cbl to four dogs is summarized in Table 1. Assimilated Cbl is defined as that fraction of Cbl that could not be removed from the intestinal segment by thorough rinsing. It is clear that the greatest assimilation of Cbl occurred in the seventh and eighth segments of the small intestine, which corresponded to the distal part of the ileum. In the proximal part of the small intestine, the ratio of luminal to mucosal Cbl was 10:1, whereas this ratio decreased significantly and reversed toward the distal small intestine even though the luminal concentration of Cbl increased, indicating that the mucosa of the ileum was selectively concentrating the vitamin. An average of 80% of the administered dose of CN[^{57}Co]Cbl was recovered in the different fractions of the small intestine.

Pooled mucosal extracts from the two most distal segments were filtered through Sephadex G-200, and five peaks (*peaks a–e*) of protein-bound CN[^{57}Co]Cbl were observed (Fig. 1). No radioactivity corresponding to free

TABLE 1. *Distribution of CN[^{57}Co]Cbl in the gastrointestinal tract of dogs*

CN[^{57}Co]Cbl	Intestinal Segment							
	1	2	3	4	5	6	7	8
A: ng assimilated/g wet wt mucosa	0.001	0.003	0.005	0.01	0.39	0.67	3.6	2.97
B: ng/ml undiluted juice	0.01	0.02	0.02	0.02	0.03	0.06	0.07	0.08
Ratio *B/A*	10.1	6.7	4.0	2.0	0.08	0.09	0.02	0.03

Measurements were made 3–5 h after the oral administration of 0.1–3.0 μg cyano[^{57}Co]cobalamin (CN[^{57}Co]Cbl) to fasting dogs (n = 4).

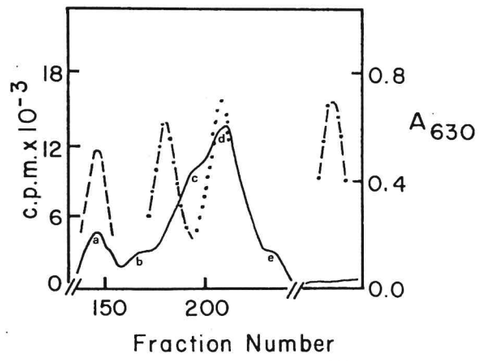

FIG. 1. Chromatography on Sephadex G-200 of a representative extract (n = 5) of dog ileal mucosa prepared (see MATERIALS AND METHODS) 3–5 h after oral administration of 0.1–3.0 μg cyano-[^{57}Co]cobalamin (CN[^{57}Co]Cbl). Marker proteins were ^{125}I-labeled immunoglobulin G (●—●—●) and human serum albumin (●●●). Blue Dextran (---) and ^3H_2O (●--●--●) indicate excluded and total column volumes, respectively. CN[^{57}Co]Cbl is shown as *solid line*.

CN[^{57}Co]Cbl appeared, confirming that all the tissue Cbl was protein bound. *Peak a* appeared in the total excluded column volume (V_o) fractions and corresponds by position to the receptor IF·Cbl complexes (25, 29). This peak constitutes somewhat more than 10% of the total bound radioactivity. After this receptor IF·Cbl complex, another radioactive peak appeared (*peak b*) before the IgG marker protein, indicating that its molecular mass was greater than 165,000 daltons. The remaining protein-bound fractions of CN[^{57}Co]Cbl, appearing as *peaks c–e*, had respective Stokes radii of 4.35, 3.54, and 2.58 nm, which were calculated as reported previously (25, 29).

Five to ten of the most radioactive fractions constituting each of these peaks were pooled, concentrated by ultrafiltration, and subjected to a number of experiments; pooled chromatography fractions from *peaks a* and *b* were filtered through Sepharose 4B columns. The radiochromatogram in Fig. 2 shows that these complexes could be resolved within this column volume into three distinct peaks having the respective molecular radii of 32.3, 12.9, and 7.8 nm. The same experiment was repeated with the addition of 1/5 vol 10 mM ethylenediaminetetraacetic acid (EDTA) or 500 mM acetate buffer (pH 4.0) into the receptor sample and then filtered through Sepharose 4B containing, respectively, 1 mM EDTA and 50 mM acetate. The first and second eluting peaks, which previously comprised more than 80% of the ^{57}Co radioactivity applied onto the column (Fig. 2), were now almost indiscernible, and there was a concomitant elution of a ^{57}Co radioactive peak after the IgG marker corresponding by position to IF. The two first eluting peaks, therefore, represent forms of the receptor-bound IF·CN[^{57}Co]Cbl, because they are EDTA and low pH inhibitable and also correspond by position (Fig. 2) to the analogous receptor complexes observed when human IF·CN[^{57}Co]Cbl coupled to the solubilized dog (29), hog (24), and guinea pig (10) IF receptor were filtered through Sepharose 4B. In all likelihood, the third peak eluting in the region before the IgG marker (Fig. 2) represents CN[^{57}Co]Cbl·R protein complex, because it did not dissociate after the addition of 1/5 vol 10 mM EDTA (data not shown) and corresponds by position to a form of R protein observed previously in extracts of hog ileal mucosa (22) (see DISCUSSION).

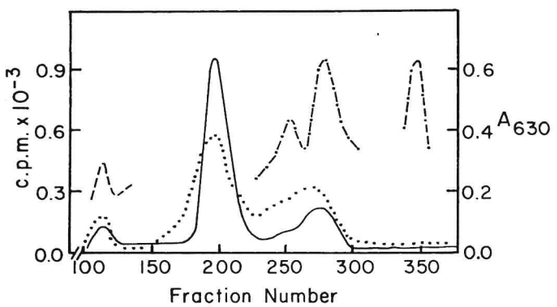

FIG. 2. Chromatography on a Sepharose 4B column (2.5 x 100 cm) of in vivo-formed dog receptor·dog IF·CN[^{57}Co]Cbl complex (●●●). These receptor complexes eluted in region between total excluded column volume and immunoglobulin G (IgG) of Sephadex G-200 column, as described in Fig. 1. Fractions comprising these receptor complexes were pooled, concentrated by ultrafiltration, and then applied to Sepharose 4B column. For comparative purposes, an elution profile of dog receptor·human IF·CN[^{57}Co]Cbl complex is given as a superimposed *solid line*. This latter receptor complex was first formed by in vitro incubation of dog ileal extract with human IF·CN[^{57}Co]Cbl and then purified by Sephadex G-200 fractionation, as similarly described for preparation of in vivo-formed receptor complex. Marker proteins were apoferritin and IgG, both labeled with ^{125}I (●—●—●). Blue Dextran (---) and $^{3}H_2O$ (●--●--●) indicate total excluded and total column volumes, respectively.

The findings described above were obtained with the 100,000-*g* supernatant fraction of the ileal mucosa homogenate after the first mechanical disruption of the mucosa from the dogs fed CN[^{57}Co]Cbl. Interestingly, the particulate material obtained from that 100,000-*g* centrifugation contained more than 40% of the total ^{57}Co radioactivity that had accumulated in the ileal mucosa before the animals were killed. After the three sequential homogenizations and centrifugations at 100,000 *g* of the initial pellet using shearing forces as described under MATERIALS AND METHODS, the final particulate fraction still contained more than 36% of the initial total ^{57}Co radioactivity. Virtually all of the residual radioactivity in the pellet was solubilized by the final extraction procedure using buffer containing Triton X-100. A sample of this supernatant was analyzed by Sephadex G-200 chromatography, and a single major well-defined symmetrical peak of ^{57}Co radioactivity was eluted from the column in the region of HSA followed by a second minor ^{57}Co radioactivity peak corresponding by position to TC-II (Fig. 3). The major peak had an estimated (29, 30) molecular radius of 3.54 nm. Approximately 8% of the total ^{57}Co radioactivity eluted into the region of the V_o and corresponded by position to the receptor observed in the experiment that is illustrated in Fig. 1.

The fractions comprising the major peak of the protein-bound CN[^{57}Co]Cbl were pooled and concentrated by ultrafiltration under negative pressure and then incubated separately with 2 ml of fresh ileal IF receptor extract prepared as described previously (29) and with 1 ml of anti-IF serum. Each of these incubation mixtures was then filtered separately through a Sephadex G-200 column. The ^{57}Co radioactivity of the sample incubated with IF receptor before chromatography eluted as it did in the absence of the receptor with an estimated molec-

ular radius of 3.54 nm (Fig. 3). Most of the ^{57}Co radioactivity of the sample incubated with the antiserum eluted in the region between the V_o and IgG marker, indicating (29) that the Cbl was still complexed to a protein that is immunologically identical with IF.

DISCUSSION

Muyshondt and Schwartz (31) have previously shown that total gastrectomy in the dog reduced the absorption of radioactive Cbl to less than 1% of the administered dose and that this was corrected by the concomitant administration of IF. In addition, a long series of reports by Abels and Muckerheide (1, 2) demonstrated that *1*) some pernicious anemia serums block binding of Cbl to dog gastric juice and *2*) antiserum to dog Cbl-binding proteins can reduce appreciably the Cbl absorption in dogs. We have now extended these observations by directly demonstrating the in vivo binding of Cbl by IF and other binding proteins in the alimentary tract of the dog and the association of the IF·Cbl complex with the receptor protein in the ileal mucosa (Fig. 1).

When CN[^{57}Co]Cbl was administered in physiological amounts to dogs, it accumulated in the intestinal mucosa in an increasing gradient extending from the proximal to the distal segments of the small intestine, which correspond to the known specialized sites for IF-mediated absorption of this vitamin in humans (6) and other animals (3) including the dog (5). It is thermodynamically unlikely (36) that Cbl is absorbed by mass action or any mechanism not involving carrier proteins, simply because all of the vitamin in the lumen and the mucosa was found to be protein bound and the molar concentration of the assimilated CN[^{57}Co]Cbl in the mucosa was substantially greater than in the intestinal succus.

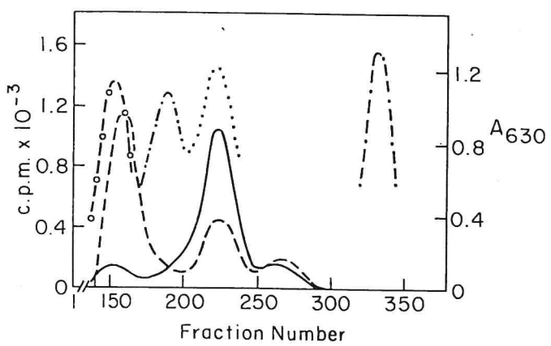

FIG. 3. Chromatography on Sephadex G-200 of mucosal extract prepared with Triton X-100 after oral administration of cyano-[^{57}Co]cobalamin (CN[^{57}Co]Cbl) (*solid line*). Note that major fraction of Cbl eluted in region of human serum albumin (HSA) and corresponds by position to intrinsic factor (IF) (cf. Ref. 29). In a subsequent experiment in this same column, fractions constituting major peak in region of HSA and its shoulder (*solid line*) were pooled, concentrated, and incubated with anti-IF serum and then rechromatographed. In this instance (---), major fraction of ^{57}Co radioactivity shifted into region of totally excluded column volume, indicating that Cbl binder in region of HSA (*solid line*) was IF. Marker proteins were ^{125}I-labeled immunoglobulin G (●—●—●) and HSA (●●●). Blue Dextran 2000 (○-○-○) and $^{3}H_2O$ (●-●-●) indicate excluded and total column volumes, respectively.

After the oral administration of CN[^{57}Co]Cbl, the extract of the distal ileal mucosa was found to contain macromolecular complexes of the vitamin. Determination of the molecular size, reaction with anti-IF serum, and dissociation of the receptor-bound IF·Cbl in EDTA and low pH buffers clearly showed that a substantial amount of the vitamin was bound to IF and the IF receptor protein, supporting the conclusion that Cbl absorption in this animal is IF mediated, as it is in all other carnivores studied (see reviews in Refs. 3, 12, 23).

The previous failure (see introduction section) to demonstrate enhanced uptake of Cbl by ileal loops containing dog gastric juice saturated with radioactive Cbl was most likely due to the low concentration of IF and high concentration of R proteins in the gastric secretions of this animal. IF comprises less than 10% of the unsaturated Cbl-binding capacity in dog gastric juice (29), and the relative preponderance of R protein(s) most certainly could obfuscate the significance of so small a fraction of total Cbl that could couple to the ileal mucosa in such experiments. This excess of R protein-bound Cbl does not impair the physiological transit of Cbl from stomach to distal ileum, because R protein(s) contained in saliva and gastric secretions [and other non-IF Cbl-binding protein(s) contained in bile] are enzymatically degraded in the intestinal succus by pancreatic enzymes (4, 11, 28).

The criteria for identification of IF in the present studies were *1*) reaction with specific type-II anti-IF antibody, *2*) molecular size and radius of the protein, and *3*) EDTA and low pH dissociation from complexes with the receptor protein formed in vivo. The observed cross-reaction of canine IF with antiserum to human IF was an unexpected observation in view of the previous studies that did not demonstrate such immunologic homology of IF in these two species (17, 37–39). This apparent discrepancy, however, can be reconciled as follows. First, the very low concentration of IF relative to the total unsaturated Cbl-binding capacity in gastric juice might have obfuscated the blocking effect of the antihuman IF antiserum when radioimmunoassay alone was used for this assessment. As a matter of fact, Yamaguchi et al. (39) found that up to 8.5% of the total Cbl-binding capacity of dog gastric juice could be blocked with such antiserum; yet, these investigators concluded that dog gastric juice contains no IF. Second, the lack of reaction of canine IF with antihuman IF antibody was demonstrated only by the blocking effect of the antiserum on the coupling of Cbl to IF. These two heterologous IF molecules, however, may still share some common antigenic determinants that are remote from the Cbl-binding site and can only be assessed, as in the present studies, with the heterogeneous population of multideterminant binding-type antibodies (32).

In the present studies, the methodology to identify IF immunologically could measure precisely and specifically immunoreactive IF, inasmuch as the immune IF·antibody complexes were clearly separated from all other unreacted Cbl·protein complexes by column chromatography. The possibility that these immune complexes, which were totally excluded from the column, might represent nonspecific aggregation of Cbl binders or Cbl coupled to serum-binding proteins is untenable because

1) the canine Cbl-binding proteins did not form such macromolecular complexes in the absence of specific anti-IF serum or IF receptor (29); and *2*) all Cbl in the ileal extract was found to be clearly coupled to proteins (see RESULTS, and Fig. 1), and no free Cbl was available to bind to serum proteins.

The ileal mucosal extract contained, in addition, Cbl bound to a protein with a molecular weight of 39,000, which does not significantly differ from the 38,000–40,000 value previously estimated for the canine TC-II (9, 33). This 39,000-dalton protein absorbed to QUSO G-32, a property also shown to be specific for TC-II (19).

The identification in the ileum of this protein, with physical properties similar to TC-II only after oral administration of CN[^{57}Co]Cbl and not when the vitamin was added directly to the ileal extract (29), suggests a physiological relation between the process of Cbl absorption and the formation of TC-II·Cbl complex. If the TC-II had simply contaminated the ileal extract from the extracellular fluid, then this complex should have been identified in both preparations. The close association of TC-II with Cbl absorption suggested by these experiments is consonant with other recent studies: in the guinea pig, where TC-II·Cbl appeared in the medium and extracts of segments of ileum in short-term culture (35); in humans, where saturation of circulating plasma TC-II with CN[^{57}Co]Cbl failed to suppress the appearance of CN[^{58}Co]Cbl bound to TC-II after oral administration of the latter radioisotopic form of the vitamin (7); and in the rat, where Cbl·TC-II appeared in the in vivo perfusate of rat small intestine after intestinal absorption of the vitamin (21).

The IF·CN[^{57}Co]Cbl that was identified in the mucosal extract unattached to the receptor protein may have been a contaminant from intestinal juice, although the mucosa was thoroughly washed before homogenization and the relative concentration of this peak was greater than would have been expected of a contaminant. Another possibility is that this IF·Cbl was released from receptor complexes during the extraction procedure. Finally, this complex may represent IF·Cbl that has been transposed to an intracellular position during the process of absorption. A previous in vivo study (34) in the guinea pig has demonstrated the accumulation of Cbl bound to a protein with immunologic properties of IF in the 10,000-*g* subcellular fraction of the ileal mucosa, and two recent studies (20, 35) using in vitro tissue culture also indicate that the IF·Cbl complex enters the ileal mucosa. The membrane-bound fraction of IF·CN[^{57}Co]Cbl, which solubilized from mucosal extract with Triton X-100 and retained its immunoreactivity with anti-IF antiserum but no longer reacted with receptor protein, may be a modification of the IF molecule that occurs during transepithelial transport. The fact that this detergent-solubilized immunoreactive IF was still bound to CN[^{57}Co]Cbl supports the notion that this IF molecule was the effector transport protein that initially attached to the receptor protein on the microvillus membranes on the surface of the mucosal cells. It is also possible that the presence of Triton X-100 has somehow damaged the structural integrity of the IF molecule, thus rendering it biologically inactive; however, this is unlikely because, as demonstrated previously, this detergent does not bind to proteins and preserves all biological properties of functional molecules (15). Moreover, more specific studies with Cbl-binding proteins demonstrated that Triton X-100 does not interfere with the coupling of the IF·Cbl to the receptor protein (25, 26).

Another protein·Cbl complex (*peak b* in Fig. 1) that was observed in the intestinal extract had a molecular weight greater than other previously identified R proteins (23). The mass of this complex was smaller than the receptor complexes, and it did not dissociate in the presence of EDTA or at low pH, indicating that this was not another form of receptor protein·IF·Cbl complex. The exact nature of this protein·Cbl complex must be more definitively established, but a similar Cbl·protein complex has been previously observed in the hog, and it was identified immunologically and isoelectrically as R protein (22). The identification of this large-sized Cbl·protein complex in addition to the smaller 104,000-dalton protein·Cbl complex in gastric juice as R proteins is consistent with the known heterogeneity of this class of Cbl binders. In fact, R proteins differ in molecular weight, isoelectric points, and carbohydrate content in tissues and fluids even in the same animal (3, 12, 14, 23, 27).

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