Cobalamin Malabsorption Due to Nondegradation of R Proteins in the Human Intestine

INHIBITED COBALAMIN ABSORPTION IN EXOCRINE PANCREATIC DYSFUNCTION

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ABSTRACT In vivo studies demonstrate that the pancreatic enzymes and the ionic environment in the upper gastrointestinal tract are essential determining factors for transport and absorption of cobalamin in man.

Jejunal fluid was aspirated from healthy human volunteers after administration of cyano[^{57}Co]cobalamin preparations. Immunochemical analysis of the aspirates demonstrated that all isotopic vitamin was transferred to a protein that is identical to the gastric intrinsic factor in terms of molecular mass (57,500), ionic nature (mean pI, 5.09), and reactivity with anti-intrinsic factor sera. However, in the aspirates from patients with exocrine pancreatic dysfunction the vitamin was found to be coupled >60% to a protein identical to R proteins in terms of molecular mass (125,000), ionic nature (mean pI, 3.51), and reactivity with anti-R protein and anti-intrinsic factor sera. The preferential transfer of cobalamin to R proteins in the patients and to intrinsic factor in healthy subjects was associated, respectively, with low and normal levels of pancreatic enzymes in the intestine and these in turn were paralleled respectively by impaired and normal ileal absorption of cobalamin. These findings confirm the suggestion that the formation of unabsorbable cobalamin complexes may be the reason of impaired vitamin absorption in exocrine pancreatic insufficiency.

Observations made with other selected patients demonstrate: (*a*) that decreased enzyme activity and nondegradation of R proteins may also be due to nonactivation of pancreatic zymogens in an acidic pH of the intestinal juice and; (*b*) that in the absence of an acidic environment in gastric juice the vitamin transported to the jejunum couples to intrinsic factor when pancreatic function is normal, and to intrinsic factor and R protein in exocrine pancreatic insufficiency. The observations made with these selected patients may explain why not all patients with exocrine pancreatic insufficiency develop impaired cobalamin absorption, and also why the malabsorption is corrected by the administration of bicarbonate in certain patients.

INTRODUCTION

A common feature of the untreated exocrine pancreatic insufficiency (EPI)[1] is the impaired ileal absorption of cobalamin (Cbl; vitamin B_{12}) (1–8). The mechanism(s) underlying the pathophysiology of this phenomenon have not been worked out (8–12). The two principal prerequisites for normal Cbl absorption i.e., the complexing of dietary Cbl to the gastric intrinsic factor (IF) (gastric phase of Cbl transport), as well as the subsequent attachment of the latter complex to specific receptors located on the ileal enterocyte (ileal wall phase) (13–15), have not been found to be directly related to parameters of the exocrine pancreatic function. Consequently the malabsorption of Cbl in EPI may not be considered in terms of events occurring during the gastric and ileal wall phase of Cbl transport. It appears likely: (*a*) that the impairment of Cbl absorption in EPI is a result of a biochemical

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[1] *Abbreviations used in this paper*: EPI, exocrine pancreatic insufficiency; HSA, human serum albumin; IF, intrinsic factor; V_o, totally excluded column volume.

defect that supervenes somewhere between the gastric and the ileal wall phase of the Cbl transport, i.e., during the intraluminal phase of Cbl transport and; (*b*) that this defect does not alter the function of IF as such but rather the mode of transport of Cbl itself. These possibilities have recently been fully appreciated by Allen et al. (16), who speculated that the presence of undegraded R-type Cbl-binding protein(s) (R protein,[2] cobalophilin, non-IF, etc.) in the intestinal lumen can inhibit the ileal absorption of the vitamin, and showed by in vitro studies that purified pancreatic proteases can degrade R proteins but not IF (16). In addition, other concurrent (14) and more recent in vivo studies (17) demonstrated that IF free of Cbl remains biologically, immunologically, and physicochemically unaltered, whereas the R protein(s) are being degraded during normal intraluminal transport. These latter observations supported the thesis (16) that in EPI the R protein(s) are not subject to degradation and therefore the entire fraction of Cbl bound to these nonfunctional proteins will not be absorbed (14, 16, 17). Recent studies in Rothenberg's laboratory (18) indicated that bile effectively sequesters Cbl from IF to nonfunctional binders and that this process can be reversed after treatment with proteases (18). Other investigators envisage entirely divergent pathologic processes in EPI including an enzymatic activation mechanism for IF (19, 20) and a degradation process for nonfunctional binding proteins by pepsin (21). Nonetheless, for 45 yr speculations on the mechanisms leading to Cbl malabsorption in EPI have been made and they are numerous; reference to each one of them has been made in recent and old reviews covering various areas of the Cbl transport (*vide supra*). The present paper describes the mode of intraluminal transport and ileal wall absorption of Cbl in EPI and in control subjects, and demonstrates that the malabsorption of the vitamin in EPI is associated with the presence of undegraded formations of Cbl with R proteins. In view of these findings and also because Cbl formations with proteins other than IF are known to inhibit the ileal absorption of the vitamin, the conclusion will be drawn that the malabsorption of Cbl in EPI is indeed resultant of the failure to degrade R proteins, as suggested by Allen et al. (16).

METHODS

Materials

Radioactive compounds and other reagents. CN[^{57}Co]Cbl (215 Ci/g), CN[^{58}Co]Cbl (3.2 Ci/g), [^{125}I]iodide (100 mCi/ml) and ^{3}H-water ([^{3}H]$_2$O) (5 Ci/ml) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. ^{125}I-pteroylglutamic acid (250 Ci/mmol) was purchased from Clinical Assays Inc., Div. of Travenol Laboratories, Cambridge, Mass. Crystalline Cbl, purified human immunoglobulin G(IgG), and human serum albumin (HSA) were obtained from Fluka AG, Buchs, Switzerland. Phenylmethylsulfonyl fluoride and aprotinin (Iniprol) were obtained from Sigma Chemical Co., St. Louis, Mo., and Laboratoire Choay, Paris, France, respectively. Pentagastrin (Peptavlon) was obtained from Imperial Chemical Industries Limited, Macclesfield, Cheshire, England. Ampholines, 40% (wt/vol), were purchased from LKB Instruments, Bromma, Sweden, and Blue Dextran 2000 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Biological fluids. Gastric juice was collected on ice from subjects undergoing general gastric analysis, depepsinized by pH adjustments (22), centrifuged at 30,000 *g* for 30 min, and stored at −80°C. A pool of sera was prepared from blood collected from patients with pernicious anemia having high titers of anti-IF type II antibodies. Threefold excess nonradioactive Cbl was added into these sera and the unbound Cbl was removed by dialysis against 0.1 M phosphate buffer, pH 7.4 adjusted to contain hog pyloric Cbl-binding preparation (23) of an unsaturated Cbl binding capacity corresponding to 2 ng Cbl/ml of buffer. The rabbit serum reported by Burger and Allen (24) and described to contain specific anti-human R protein antibodies was kindly prepared and sent to us in lyophilized state. The serum had unsaturated Cbl binding capacity and this was completely masked with nonradioactive Cbl as described above for the anti-IF sera. The absence of free Cbl binding sites in the Cbl treated antisera was confirmed by determinations for unsaturated Cbl binding capacity using albumin-coated charcoal.

Intubation of patients. A triple lumened naso-gastrointestinal tube was used for the administration to the patients of various preparations of Cbl as well as for the aspiration of juices. The tube had been devised in the laboratory of Dr. Matuchansky (Poitiers, France) and modified by us and adapted to the requirements of the present studies. In its final form, it consisted of three tubes assembled together with cyclohexanone. The first tube (naso-gastric tube) measuring 103 cm had an internal diameter of 2 mm, opened into the gastric antrum, and was used to instill the various Cbl preparations into the gastric succus. The second tube (nasointestinal tube) measuring 150 cm had an internal diameter of 2 mm, was fabricated with radioopaque, polyvinyl chloride (Portex Limited, Hythe, Kent, England), and opened into the proximal jejunum in the region below the duodenojejunal flexure. The distal 3-cm part of the tube bore several added side holes through which the intestinal samples were aspirated. The third tube, also fabricated with polyvinyl chloride, had a diameter of 1 mm and opened slightly beyond the nasointestinal tube described above and was equipped with a rubber condom filled with 3 ml 99.9999% pure mercury (code 25.359.18 Prolabo, Rhône-Poulenc, Paris, France). This tube was used for air insufflation by a syringe into the intestinal lumen to prevent the obstruction of the nasointestinal tube by the jejunal mucosa that was sucked in. The patients were fasted for 24 h after intubation and this was followed by fluoroscopic examination to ensure that each tube had been properly placed in position.

Procedures

Collection and preparation of intestinal juices. Informed consent was obtained from control subjects and patients (see Subjects) in accord with the Declaration of

[2] The term "R protein" was originally devised to denote a B_{12}-binding protein in human gastric juice that was devoid of intrinsic factor activity, but the same term is currently used for other immunologically related proteins present in most biologic fluids and body tissues.

Helsinki after approval by the Medical Faculty of the University of Nancy I. Patients receiving preparations of pancreatic extract were hospitalized 1 d before intubation and did not receive any additional supplement. The regular treatment of these patients was resumed immediately after the samples of intestinal juices had been collected as described below.

40 min before the administration of Cbl preparations (see below) to each subject a 20-ml blood sample was collected followed by aspiration of residual contents through the nasointestinal tube. This was followed by subcutaneous injection of pentagastrin, 6 μg/kg body wt followed 20 min later by administration through the naso-gastric tube, of a test meal composed of 25 g corn oil, 30 g of protein mixture (LPS 85 Gerble, Revel, France) containing 25.5 g milk proteins, 0.6 g lipid, 1.5 g lactose, and 1 g various salts but no Cbl, and 345 ml distilled water. The pH of this unbuffered suspension was in the range of 7.2 to 7.6.

The Cbl preparation (250 ng CN[^{57}Co]Cbl, 2 μCi) (range 1.3–2.1 μCi) in free form or coupled 53% to IF and 47% to R protein in normal human gastric juice was administered to the patients 2 min before the administration of the test meal. Concurrently with the administration of the Cbl preparation, samples were aspirated continuously through the nasointestinal tube and collected on ice. A gamma scintillation detector polyradiometer IPAB-71, type E500, Nardeux, Loches, France, was used to monitor the radioactivity of the samples withdrawn from each patient. After measurements of pH and radioactivity, the fractions were pooled and processed as described below.

Except when otherwise stated in the text, a 20-ml sample for enzyme activity measurements from each pool was kept at −80°C without any further treatment. The remaining of each pool was depepsinized by pH adjustments and centrifuged at +4°C and 30,000 g for 1 h to remove particulates. Each pool was mixed with ¼ vol of 0.1 M phosphate buffer, pH 7.4, containing 0.02% (wt/vol) NaN_3, 0.02 mM phenylmethylsulfonyl fluoride, 5,000 U aprotinin/liter, and 0.154 M NaCl.

Schilling procedure. All subjects were rehospitalized ~10–30 d after the collection of the respective intestinal juices. 1 d later, ileal absorption of Cbl was evaluated by the Schilling procedure (25). Patients receiving oral administration of preparations of pancreatic extract were asked to stop their treatment upon hospitalization and restarted after the collection of the 24-h urine samples. All subjects remained fasted overnight before administration of the radioactive mixtures on the subsequent morning. Doses of 250 ng of CN-[^{57}Co]Cbl were given in free form or bound to human gastric juice each preceded by an injection of 1 mg nonradioactive Cbl (Delagrange, Paris, France) i.m. The absorption was then measured with the use of two sequential 24-h urine collections (25, 26).

Assays. Unsaturated Cbl binding capacity was measured using albumin-coated charcoal (27). The content of IF and R protein in gastric juice was measured using the type II (28) anti-IF sera. Cbl concentration was measured by competitive radioassay in sera denatured after boiling at low pH in the presence of cyanide using purified hog IF as binding protein (29). Folate concentration was measured also by competitive radioassay in sera denatured after boiling, using ^{125}I-pteroylglutamic acid as tracer and bovine milk folate binder as binding protein (30). Chymotrypsin activity in 0.1 ml samples of intestinal juice was measured (31) spectrophotometrically at 256 nm at pH 7.8 and 25°C using incubation with 0.5 mM *N*-benzoyl-L-tyrosinethyl ester in 50% (wt/vol) methanol as substrate in the presence of 50 mM Ca^{2+}. Molar extinction coefficient used for *N*-benzoyl-L-tyrosine was 964. Lipase activity in the intestinal juice was measured by potentiometric titration from the rate of hydrolysis of the glycerides contained in an olive oil emulsion at pH 8.0–8.2 essentially as described (32).

Chromatographic procedures. Isoelectrofocusing. Isoelectrofocusing in standard 110-ml LKB columns type 8101, was carried out at +4°C. Carrier ampholines of pH 4.0–6.0 and 2.5–5.0, in a final concentration of 1% (wt/vol) and in 0–50% (wt/vol) sucrose (sucrose for density gradient ultracentrifugation, Merck AG, Darmstadt, West Germany) gradient, were used for electrofocusing the IF and the R protein preparations, respectively (23). 380 and 500 V were applied for the first 3-d and the 4th d period, respectively.

Molecular sieve gel chromatography. Columns, 2.5 × 100 cm, were packed with Sephadex G-200 (Pharmacia Fine Chemicals) and equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 0.154 M NaCl and 0.02% (wt/vol) NaN_3. Packing and elution of columns was carried out at +4°C at a constant flow of 11.5–12.0 ml/h. Fractions of 1.0–2.0 ml vol were collected (23).

Protein markers and other compounds used for estimation of molecular characteristics. Purified IgG and HSA were labeled with ^{125}I using chloramine-T (33). Continuous dialysis against 25 mM phosphate buffer, pH 7, containing 7.5 g/l Bio-Rad AG-1 X 8 (Bio-Rad Laboratories, Richmond, Calif.) was used to remove free iodide (34). Blue Dextran 2000 and $[^3H]_2O$ were used for estimating the totally excluded column volume (Vo) and the total volume accessible to the solvent.

Purification procedures. Purified R protein and IF complexed to CN[^{57}Co]Cbl were prepared from intestinal samples collected from patients with exocrine pancreatic insufficiency and healthy subjects, respectively, using incubation with specific antisera and Sephadex G-200 filtration as described previously (34).

Computations of molecular mass by gel filtration. The molecular mass of the IF-CN[^{57}Co]Cbl and R protein-CN[^{57}Co]Cbl complexes was estimated as described recently (34) using the formula illustrated below:

$$M_x = \text{antilog}\,[(\log M_2 - \log M_1) \times (K_{d1} - K_{dx})/(K_{d1} - K_{d2}) + \log M_1],$$

where M = molecular mass, K_d = distribution coefficient. The subscript x denotes the unknown protein; 1 denotes the first eluting marker protein (here IgG; M_1 = 165,000) and 2 denotes the second eluting marker protein (here HSA; M_2 = 69,000).

Subjects

Controls. The subjects referred to in the text as controls were normal in the sense that they had no evidence for EPI, showed no calcification in the upper gastrointestinal series, had no steatorrhea or diarrhea, and all serum parameters and other chemistries related to exocrine pancreatic function were normal, except as otherwise stated in the text. Thus controls I, VI, and VIII had entirely negative medical histories, but all the other controls (II–V and VII) had various minor medical problems.

Patients. Patients I to VIII represent subjects with proven EPI. The diagnosis had been based primarily on a positive medical history including pancreatic calcifications, steatorrhea, findings obtained using ultrasonography or computerized tomography and absence of pancreatic enzyme activities in the jejunal fluid or a combination thereof (see Table I).

Selected patients. The salient features of the medical histories of a patient with proven EPI (patient VI) and two non-EPI patients (patients IX and X) are given here because

TABLE I
Tabulation of Clinical and Laboratory Data of All Subjects Studied

Patients with exocrine pancreatic insufficiency	Pancreatic calcifications	Fat in stool	Enzyme activity in intestinal juice		Serum Cbl	CN[^{57}Co]Cbl excreted into urine		Mode of transport of CN[^{57}Co]Cbl*	
			Lipase	Chymotrypsin		Stage I‡	Stage II‡	Intrinsic factor	R protein
		g/d	*U/ml*		*ng/liter*	*% total oral dose*		*%*	
I	+	12.4	0.00	0.00	1125	1.60	1.80	21.6	78.4
II	+	20.43	0.00	0.00	1200	8.90	9.50	15.6	84.5
III	–	0.8§	11.25	3.10	440	5.00	5.00	21.2	78.8
IV	–	1.0§	5.00	15.60	920	5.00	5.00	27.6	72.4
V	+	8.28	0.00	0.00	1200	0.64	0.72	21.0	79.0
VI	+	8.46	0.00	0.00	1200	7.00	10.00	53.0	47.0
VII	+	4.61	0.00	2.25	1200	13.60	14.60	86.9	13.1
VIII	?	8.27	0.00	0.00	640	6.00	7.50	48.8	51.2
Other patients									
IX	–	1.1	23.70	35.00	680	18.00	18.00	100.0	00.0
X	–	4.1	26.10	33.40	640	26.00	29.00	100.0	00.0
Control subjects									
I	–	1.08	110.00	29.50	560	30.30	33.60	100.0	00.0
II	–	4.1	10.00	26.00	325	12.70	12.70	100.0	00.0
III	–	2.3	25.00	25.00	1100	33.10	33.30	100.0	00.0
IV	–	4.35	75.00	12.50	590	20.00	20.00	100.0	00.0
V	–	0.75	35.00	13.70	780	17.70	22.00	100.0	00.0
VI	–	3.80	28.70	43.00	940	19.90	21.00	100.0	00.0
VII	–	2.15	24.00	37.50	540	12.00	16.00	100.0	00.0
VIII	–	1.2	13.75	87.50	940	33.10	33.30	100.0	00.0

* Calculated by integration of the peaks representing the intrinsic factor and R protein in Sephadex G-200 chromatography.
‡ Schilling procedure without (stage I) and with (stage II) coadministration of gastric juice.
§ Patient III and patient IV had only 37 and 92 g total stool per 3 d.

the pertinent observations made with these patients are of particular importance in our understanding of the role of pH in the modification of binding of the vitamin to IF and R protein in the intestine.

Patient VI underwent two laparotomies: pancreaticojejunostomy for drainage of pancreatic cyst, and diverting choledochogastro-anastomosis.

Patient IX was a 44-yr-old male with chronic gastric ulcer, gastritis, and duodenitis. Gastric analysis showed a basal gastric secretion of 39.20 ml during a 90-min period of collections with a mean pH value of 7.60. The corresponding figures after gastrin stimulation were 32.80 ml and mean pH 7.40. Both measurements indicated an acid output of zero. In addition, high gastrin concentration in blood of 190 pg/ml (normal 100 pg/ml) was measured.

Patient X was a non-EPI patient. An abnormally low pH was measured in his intestinal juice (see Results).

RESULTS

Magnitude of secretion, pH, and speed of transport in the upper gastrointestinal lumen. Fig. 1 illustrates the Sephadex G-200 radiochromatogram of the ^{57}Co-radioactive preparation administered to the subjects studied and shows that the protein-bound vitamin was distributed 47% to R proteins and 53% to IF. After the administration of the radioactive preparation, the specific activity of consecutive intestinal samples aspirated from each subject (see Methods) increased rapidly and within an average period of 16 min (extreme values, 10 and 34 min) reached a peak, and then gradually decreased over 1–3 h. The volume of intestinal juice aspirated from each patient and each control was in the range of 51 to 187 ml (mean 125 ml) and 62 to 220 ml (mean 145 ml), respectively. The average specific activity of the pooled intestinal juices from patients was 5 nCi/0.51 pmol Cbl per ml. The respective figure for control subjects was 4.8 nCi/0.55 pmol Cbl per ml. The average proportion of the total administered dose that was recovered in the aspirates was 48.1% for patient and 50% for control subjects. After centrifugation as described in Methods, the average fraction of the total radioactivity that was precipitated was 15.5 and 6.3% for patient and control subjects, respectively.

Chromatographic analysis of in vivo formed CN[^{57}Co]Cbl-protein complexes during intraluminal transport in the upper gastrointestinal tract of healthy subjects. An appropriate volume from each

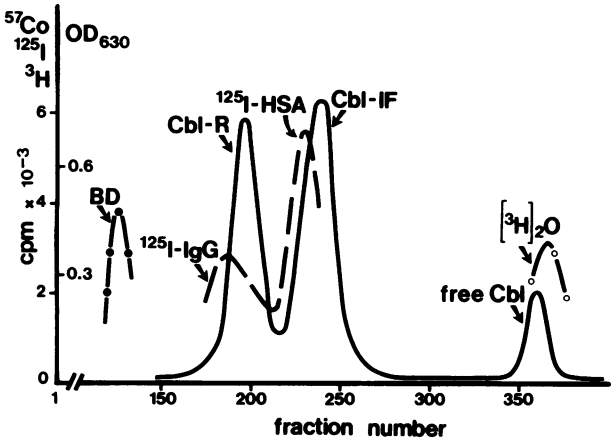

FIGURE 1 Radiochromatogram of the gastric juice preparation administered to all subjects studied. CN[^{57}Co]Cbl coupled to this gastric juice eluted from Sephadex G-200 (——) 47% bound to R protein and 53% bound to IF. (– – –), ^{125}I-IgG and ^{125}I-HSA; (·–·), Blue Dextran 2000 (BD).

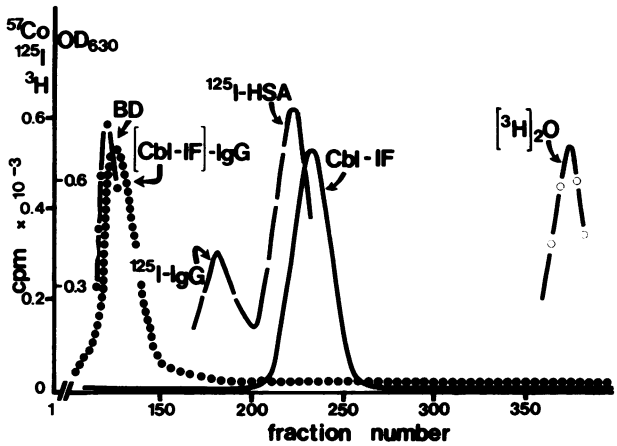

FIGURE 2 Typical radiochromatogram obtained with each one of the ^{57}Co-radioactive intestinal juices aspirated from control subjects (——). The single ^{57}Co-radioactive peak eluting after ^{125}I-HSA represents Cbl-IF complex because this peak shifted into the region of V_0 after preincubation with the pooled anti-IF serum (●).

individual pool of juices from control subjects containing 14–20 pmol CN[^{57}Co]Cbl (0.2–0.3 μCi) were concentrated to 2.0–4.0 ml final vol by ultrafiltration using negative pressure and then recentrifuged to remove precipitated material. In one series of experiments half a volume from each concentrated preparation was mixed with 1 μl [^{3}H]$_2$O diluted 1:2,000 (vol/vol) with nonradioactive water, 3 mg Blue Dextran 2000, and 72 ng of ^{125}I-labeled IgG and HSA (0.3–0.5 μCi), and then filtered through Sephadex G-200 columns. In another series of experiments the remaining half of each one of the concentrated preparations was incubated under agitation for 40 min with pooled type II anti-IF sera followed by addition of markers (see above), and then filtered. When the unincubated samples were filtered as such the ^{57}Co-radioactivity eluted in its entirety as a symmetrical well-defined peak slightly after the ^{125}I-HSA marker protein (Fig. 2), with an estimated molecular mass of 57,500 indicating that all CN[^{57}Co]Cbl was protein bound. When the samples incubated with the anti-IF sera were similarly fractionated the 57,500-dalton peak shifted from its previous position, after HSA, into the region of the V_0 corresponding to an average estimated molecular mass of 550,000 (Fig. 2). This shift of elution position indicates (23) that the 57,500-dalton peak was IF because the antibodies contained in the pooled anti-IF serum had reacted with the total CN[^{57}Co]Cbl-protein complex contained in intestinal juice.

Chromatographic analysis of in vivo formed CN[^{57}Co]Cbl-protein complexes during intraluminal transport in the upper gastrointestinal tract of patients with exocrine pancreatic dysfunction. In contrast to the Sephadex G-200 elution patterns observed with the control subjects, the ^{57}Co radioactivity from patients (patients I–VIII, only) resolved into two peaks (Fig. 3); the first eluting peak was on the average the major one, had a molecular mass of 125,000 and contained from 13 to 85% (mean 63.1%) of the total CN[^{57}Co]Cbl present in each sample; the second eluting peak contained the rest of the total CN[^{57}Co]Cbl and had a molecular mass of 57,500. When intestinal samples from patients were preincubated with an excess of pooled anti-IF type II antibodies and then filtered through Sephadex G-200, the 57,500-dalton but

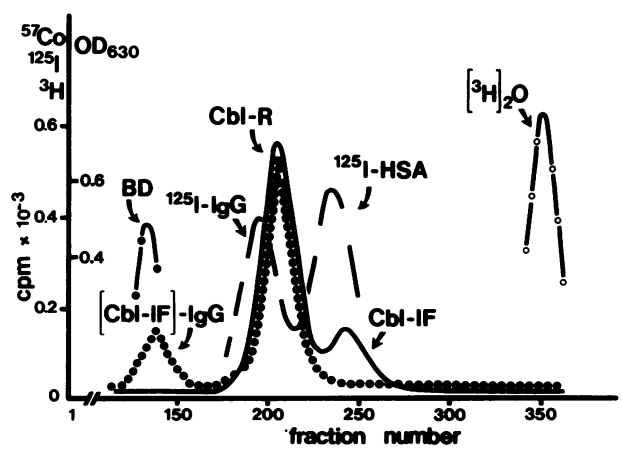

FIGURE 3 Typical radiochromatogram obtained with each one of the ^{57}Co-radioactive intestinal juices aspirated from patients with EPI (——). The ^{57}Co-radioactive peak eluting after ^{125}I-HSA represents Cbl-IF complex because this peak shifted into the region of V_0 after preincubation with the pooled anti-IF serum (●). Only variations in the relative content of IF were observed among the eight individual intestinal fluids studied (see Table I).

not the 125,000-dalton peak, shifted into the region of V_0 (Fig. 3). The repetition of this experiment using specific rabbit anti-human R protein instead of the anti-IF serum resulted in a shift of the 125,000-dalton peak into the V_0 while the 57,500-dalton peak now remained unchanged (Fig. 4). These results demonstrated that: (*a*) the 57,500-dalton peak is due to IF, and (*b*) the 125,000-dalton peak is due to an R protein.

Molecular characteristics of the purified intestinal intrinsic factor and R protein complexed to CN[^{57}Co]-Cbl. The estimated molecular mass values of IF and R protein complexed to CN[^{57}Co]Cbl were 57,600 (±2.900) (n = 12) and 124,900 (±3.700) (n = 12), respectively.

Isoelectrofocusing profiles. Purified IF and R protein complexed to CN[^{57}Co]Cbl were chromatographed separately in isoelectrofocusing columns (n = 8) (e.g., see Figs. 5 and 6). The IF-CN[^{57}Co]Cbl and the R protein-CN[^{57}Co]Cbl complexes resolved into eight and seven isoproteins, respectively. The mean isoelectric points were computed by integration of the relative IF or R protein activity contained in each isoprotein peak as a function of the corresponding isoelectric point value. The mean pI for the IF-CN[^{57}Co]-Cbl complex was 5.09 (±0.10) and for the R protein-CN[^{57}Co]Cbl complex was 3.51 (±0.10).

Approximately 4–8% of the R-protein-CN[^{57}Co]-Cbl prepared from two individual patient juices focused outside the pH range of the ampholine gradient used near the cathode. This fraction of radioactivity focused in the pH range of 6.27 to 9.30. Change of the position of electrodes resulted in similar observations. Addition of 1 ml 0.3 M phosphate buffer, pH 7.4, and absorption of unbound Cbl with 1 ml albumin-coated charcoal before counting the radioactivity contained in the fractions of this peak, did not change the magnitude or the position of the peak. Therefore, this peak represents Cbl coupled to protein, most probably R protein (9). Such an alkaline type of isoprotein does not represent a component of the native R protein (9, 35). It most probably corresponds to denatured or partially hydrolyzed protein (9, 14, 16).

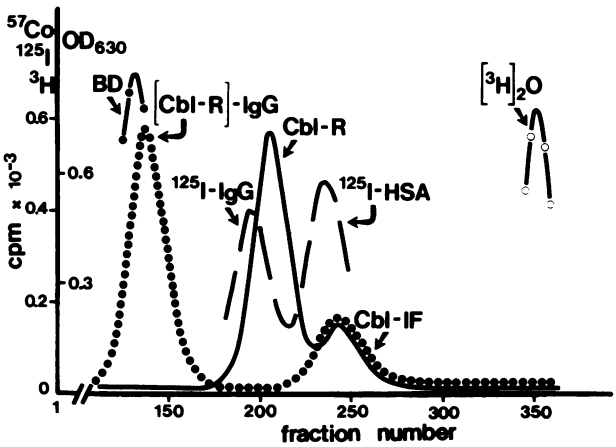

FIGURE 4 Typical radiochromatogram obtained with each one of the ^{57}Co-radioactive intestinal juices withdrawn from patients with EPI (——). The major ^{57}Co-radioactive peak eluting between ^{125}I-IgG and ^{125}I-HSA represents Cbl-R protein complex because this peak shifted into the region of V_0 after preincubation with the anti-R protein serum (●). Only variations in the relative content of R protein were observed between the eight individual patient intestinal fluids studied (see Table I).

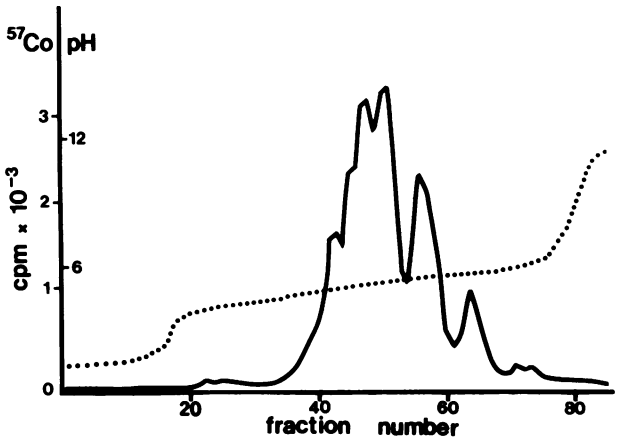

FIGURE 5 Typical isoelectrofocusing pattern (——) of IF-CN[^{57}Co]Cbl complexes purified from the intestinal fluids of EPI patients and control subjects. (·····), pH gradient.

The in vivo effect of endogenous pancreatic enzymes on intrinsic factor and R protein complexed to CN-[^{57}Co]Cbl in intestinal juice. Table I tabulates the lipase and chymotrypsin specific activity measured in the intestinal juice withdrawn from each control subject and also from patients. An average lipase activity of 40.8 U (±34.6) for control subjects and 2.30 U (±4.10)

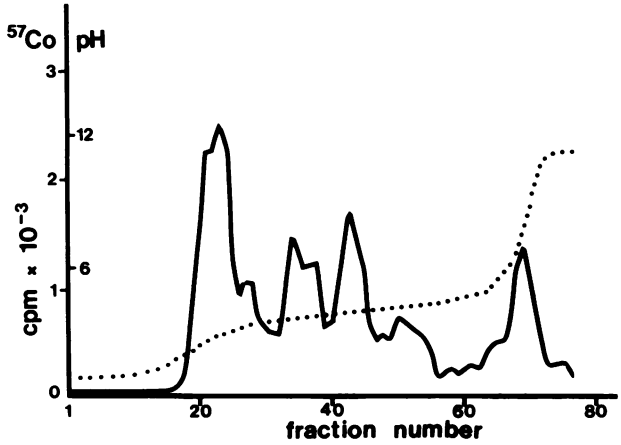

FIGURE 6 Typical isoelectrofocusing pattern (——) of R protein-CN[^{57}Co]Cbl complexes purified from the intestinal fluids withdrawn from EPI patients (·····), pH gradient.

for patients, and an average chymotrypsin activity of 34.33 U (±23.88) for control subjects and 2.62 U (±5.38) for patients, was calculated.

In retrospect, the radiochromatograms obtained in Sephadex G-200 filtration with samples from control subjects showed that 100% (±0.00%) of the CN[^{57}Co]-Cbl in intestinal juice was coupled to IF (e.g., see Fig. 2), and this is now shown to be paralleled by normal pancreatic enzyme activity in the intestine. On the other hand, it was demonstrated that an average of 63.1% (±24.4) of CN[^{57}Co]Cbl in the patient intestinal juices was carried by R proteins (see Table I), and the rest by IF, and this is now shown to be associated with almost complete absence of pancreatic enzyme activity in the intestine (see Fig. 3 and Table I).

Intraluminal transport and ileal absorption of CN[^{57}Co]Cbl. The Schilling test results demonstrated (see Table I) that the subjects with 100% CN[^{57}Co]Cbl coupled to IF in the intestinal juice had normal ileal absorption of Cbl with a mean fraction of 23.89% (±7.79) of the total oral dose excreted into the urine. On the other hand, a mean fraction of 63.1% of the CN[^{57}Co]Cbl in the intestinal juice collected from patients was found to be coupled to R protein and in these patients the absorption of CN[^{57}Co]Cbl was found to be markedly impaired with a mean fraction of only 6.76% (±4.59) of the total oral dose excreted into the urine.

Transport of CN[^{57}Co]Cbl administered in free form. In all experiments described above vitamin precoupled to gastric juice had been administered to each subject. Three additional experiments were carried out after administration of free (unbound) CN[^{57}Co]Cbl. For comparative purposes the experiments were carried out with subjects who had been preinvestigated using administration of CN[^{57}Co]Cbl coupled to gastric juice. The intestinal samples were chromatographed as described (*vide supra*) after administration of 184.5 pmol CN[^{57}Co]Cbl to control subject II and patient II (see Table I). The radiochromatograms of these analyses are presented in Fig. 7 and they are identical to those obtained when administration of CN[^{57}Co]Cbl coupled to gastric juice was used (Fig. 7 and Table I). These findings show: (*a*) that CN[^{57}Co]-Cbl instilled in free form into the gastric succus was distributed as coupled to IF and R protein in the intestinal juice in proportions similar to those formed when CN[^{57}Co]Cbl coupled to gastric juice was administered to the same subjects and; (*b*) that the mode of transport of Cbl and its fractional distribution between IF and R protein in the intestinal juice is dependent on the presence or absence of pancreatic enzymes in the intestinal juice and not to the form of administration. A third sample containing 184.5 pmol CN[^{57}Co]Cbl coupled to gastric juice and 5,166 pmol of CN[^{57}Co]Cbl in free form was administered

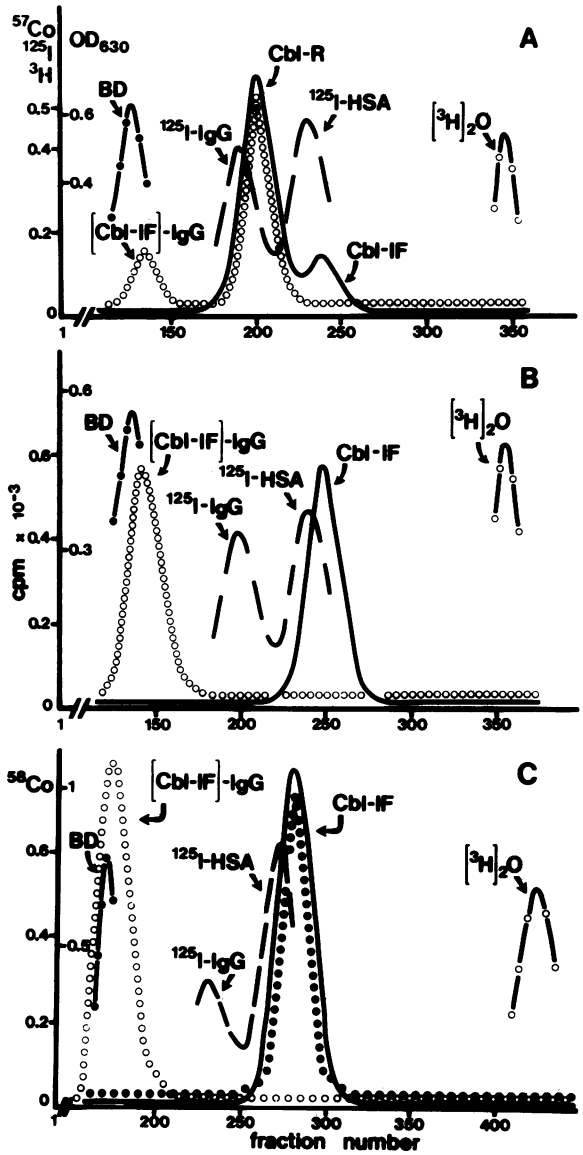

FIGURE 7 Radiochromatograms observed with ^{57}Co-radioactive intestinal juices aspirated after preadministration of 184 pmol free (uncoupled to gastric juice) CN[^{57}Co]Cbl (——) to patient II(A) and control subjects II(B) and VIII(C). Additional 5,166 pmol of CN[^{58}CN]Cbl (●, C) had been administered simultaneously with the CN[^{57}Co]Cbl to control subject VIII. (○), respective radiochromatograms observed after each one of the samples described above had been preincubated with pooled anti-IF serum and then filtered.

to control subject VIII. This aimed to demonstrate whether or not differences in the mode of transport could be induced by the administration of high concentrations of Cbl. In this instance both radioisotopes eluted in a similar manner, and both the protein precoupled CN[^{57}Co]Cbl and the free CN[^{58}Co]Cbl were found to be in their entirety bound to IF, and there was no free Cbl (Fig. 7C) in the samples.

CN[^{57}Co]Cbl transport in a patient with neutral pH in gastric juice. All CN[^{57}Co]Cbl administered to patient IX, who had a pH of 7.4–7.6 in gastric juice (see selected patients), was found to be coupled to IF in the intestinal juice (radiochromatogram similar to Fig. 2), and the ileal absorption of the vitamin was normal with a fraction of 18% of the total oral dose of CN[^{57}Co]Cbl excreted into the urine (Table I).

CN[^{57}Co]Cbl transport in a patient with abnormally low pH in intestinal juice. The pooled fractions of intestinal juice withdrawn from patient X had an abnormally low pH; a 1-h collection of juice (108 ml) before the stimulation with pentagastrin had a mean pH 4.0 and the 1-h collection (220 ml) after stimulation had a pH of 3.1. Half volume of the intestinal juice from this patient was prepared as described in Methods but the rest of it had not been subjected to depepsinization and no proteolytic inhibitors were added to it. Duplicate Sephadex G-200 radiochromatograms of the undepepsinized ^{57}Co-radioactive intestinal juice showed that all CN[^{57}Co]Cbl previously administered to the patient had been sequestered *in situ* and remained coupled to the R protein in intestinal juice (Fig. 8A). Double repetition of this experiment after simple 20-min preincubation of the intestinal juice at 22°C and pH 8.0 before chromatography, resulted in a 100% transfer of CN[^{57}Co]Cbl to IF (Fig. 8B).

CN[^{57}Co]Cbl transport in a patient with exocrine pancreatic dysfunction and neutral pH in gastric juice. Patient VI had undergone diverting pancreatic jejunostomy and might have had no pancreatic secretion above the level of anastomosis. Second, the patient had true pancreatic insufficiency for the reasons described in Methods. This certainty about the absence of pancreatic enzymes in the proximal jejunum was confirmed by the absence of detectable chymotrypsin and lipase activity (see Table I) in intestinal juice. This case appeared to be of particular interest (see Discussion) for the patient had undergone a second diverting operation, choledochogastroanastomosis, with the anastomosis located at the level of pyloric sphincter. Therefore, one might expect relatively high or nearly neutral pH in the gastric juice of this patient. Consecutive measurements of pH in his bile-stained gastric juice revealed pH values in the range of 5.3 to 6.2. The Sephadex G-200 radiochromatogram of the ^{57}Co-radioactive juice from this patient (see Table I), showed that the ^{57}Co-radioactivity was coupled 47% to R protein and 53% to IF, i.e., in proportions similar to those of the CN[^{57}Co]Cbl preparation instilled into the gastric succus of the patient (Fig. 1).

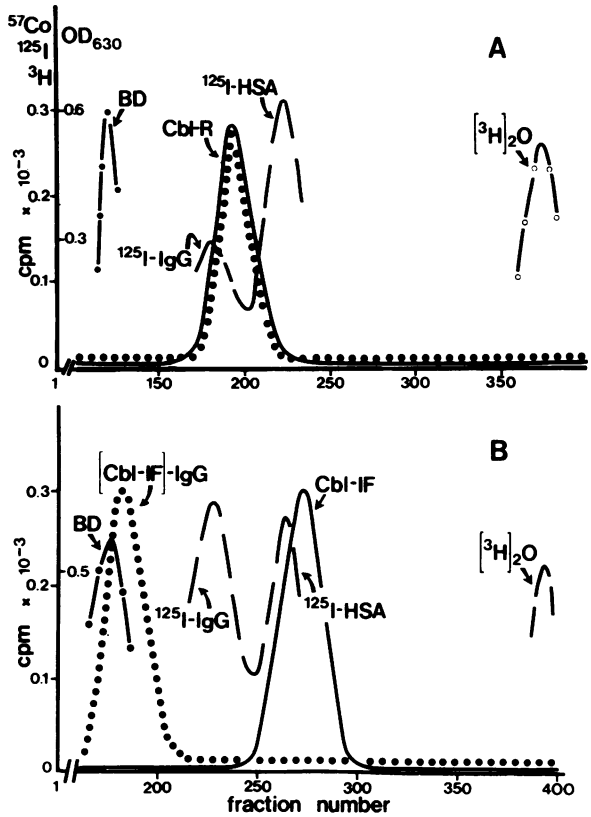

FIGURE 8 Radiochromatograms observed with the crude (unbuffered) ^{57}Co-radioactive intestinal juice aspirated from patient X (who had a low pH of 3.1 in his intestinal juice) (A, ——) and with a similar sample that had been preincubated for 20 min at 22°C and pH 8.0 before gel filtration (B, ——). (●), represent the radiochromatograms observed after the respective samples had been preincubated with pooled anti-IF serum and then filtered.

DISCUSSION

In the present studies CN[^{57}Co]Cbl coupled to IF and R proteins was instilled into the gastric succus of human volunteers. The peak of isotopic Cbl reached the jejunum within a 16-min period of transport. Chromatographic analysis demonstrated (see Fig. 2) that the CN[^{57}Co]Cbl aspirated from control subjects had been transferred to a protein that (*a*) had a molecular mass of 57,500 (see Fig. 2), (*b*) consisted of isoproteins (see Fig. 5) similar to those previously (23, 36) observed with the gastric IF, and (*c*) comprised antigenic determinants reacting with specific anti-IF antibodies. These similarities of conformation and antigenic structure demonstrated that the Cbl transported down to the jejunum was bound to IF.

The CN[^{57}Co]Cbl aspirated from the EPI patients was found to be bound mainly to a protein closely resembling the R protein(s) (see Fig. 3). The estimated molecular mass of 125,000, the positive reaction with the anti-R protein serum (see Fig. 4) and the typical R protein isoelectrofocusing profiles (see Fig. 6) demonstrated that the protein represents a typical R protein (35).

CN[^{57}Co]Cbl was administered also in free form to two control subjects (II and VIII) and to one EPI patient (II). In these instances the vitamin distribution on IF and R proteins (Fig. 7) in the respective jejunal fluids was similar to the distribution observed when the vitamin was administered coupled to gastric juice (Table I) indicating that the in vitro precoupling of CN[^{57}Co]Cbl to gastric juice can not modify the overall redistribution of vitamin on IF or R proteins in the intestine.

The fact that the CN[^{57}Co]Cbl aspirated from EPI patients was found to be 13 to 85% coupled to R protein(s) (Table I) shows that only 25–87% of the Cbl initially present in the gastric succus of these patients has prima facie the chance to be absorbed. Presumably, the unabsorbable fraction of Cbl may be as high as 95% in some EPI patients because the concentration of the IF-receptor molecules in the ileum is appreciably low (37–39), and apparently only a fraction of the IF-Cbl complex present in the ileum at any one time has the chance to gain entry into the enterocytes. Indeed, the statistical analysis (40) of the data tabulated in Table I demonstrate that as little as 5.97% (±4.1) and as much as 22.2% (±7.88) of the total oral dose of CN[^{57}Co]Cbl administered alone to EPI patients and control subjects was excreted into the urine, respectively. This finding that the presence of Cbl coupled to R protein in the intestinal juice parallels the impaired ileal absorption of the vitamin, indicates that the discrepancy in the absorption of Cbl in EPI is due to the presence of undegraded unabsorbable forms of Cbl in the intestinal lumen.

The measurements of pancreatic enzyme activity demonstrated that the EPI patients had indeed appreciably decreased secretion of pancreatic enzymes and/or zymogens in the intestine. Furthermore, the finding that the concentration of Cbl coupled to R protein was inversely proportional to the level of pancreatic enzyme activity indicates that these enzymes represent factors capable of modifying the mode of transport of Cbl in the intestine. An excellent confirmation of this suggestion was obtained in the studies performed with the non-EPI patient who had an unusually low pH in intestinal juice (patient X). 100% of the CN[^{57}Co]Cbl aspirated from this patient was found to be coupled to R proteins (see Fig. 8), presumably because of a diminished proteolytic activity at pH values < 8.0 (41) and to nonactivation of the enterokinase, trypsinogen, chymotrypsinogen, and all other nonfunctional zymogens at pH values < 6.0 (42, 43). That nonactivated zymogens were present in that intestinal juice was demonstrated by the high levels of chymotrypsin and lipase activities measured in it (see Table I) and by the 100% transfer of Cbl from R protein to IF after a 20-min incubation at pH 8.0 (see Figs. 8A and B) (44). It is not unreasonable to assume that a similar mechanism may take place in vivo and that the normal vitamin absorption observed with the patient X (Table I) was due to a late activation of zymogens after the acidity of the intestinal fluid had been neutralized by physiologic ion exchange during transport down to the ileum. Conversely, failure to neutralize the acidic jejunal juice would have left the Cbl-R protein complex behind undegraded and this would have eventually led to impaired Cbl absorption. This failure to activate the nonfunctional zymogens may account for a number of EPI cases associated with impaired Cbl absorption corrected by the administration of bicarbonates (45) and also for the unexplained impairment of Cbl absorption in patients with Zollinger Ellison syndrome (12).

The findings that 100% of the Cbl administered to patient X was transferred to R proteins in the proximal jejunum (where the pH was found to be as low as 3.1) fully confirms the suggestion that Cbl binds preferentially to R proteins in the acidic chyme of gastric secretions. However, other observations demonstrate that the pancreatic enzymes can modify drastically this initial preponderant binding of Cbl to R proteins. For instance, the pH in gastric juice of a few of the control subjects studied was presumably not sufficiently acidic because of dilution by the bulky administration of > 350 ml of the test meal solution. Furthermore, the pH of gastric juice in the hypochlorhydric patient (patient IX) was certainly not acidic. However, 100% of the Cbl administered to each one of the subjects described above had been readily transferred to IF in the intestine (Table I) indicating that the initial relative binding of Cbl to IF and R proteins or the pH in gastric juice do not bear on the subsequent overall redistribution of Cbl to IF as long as the pancreatic enzymes function normally in the intestine.

In contrast to the prevailing regulatory role of pancreatic enzymes over pH in control subjects, the pH in gastric juice appears to be the single essential determining factor for the mode of intraluminal transport and ileal absorption of Cbl in EPI. For instance, as much as 48–60% of the Cbl initially coupled to IF and administered to EPI patients (e.g., see results for patients I–IV in Table I) was redistributed to R proteins during transport down to the jejunum. Previous in vitro studies demonstrated that R proteins sequester Cbl from IF in low pH (16) and presumably the transfer of Cbl to R protein observed here with the EPI patients was also due to the low pH in gastric juice. This mechanism is consonant with the findings obtained with patient VI and can explain the absence of Cbl-sequestration in that patient (see Table I) who was also an EPI patient but with a nearly neutral pH in his gastric juice, as well as the 100% sequestration of Cbl to R protein in the non-EPI

patient X who had a pH as low as 3.1 in his intestinal juice.

The administration of test meals might have induced a slight rise of pH in the gastric juice of certain EPI patients studied, thus resulting in a slower and not complete transfer of Cbl to R proteins. A similar mechanism explaining the absence of Cbl malabsorption in certain EPI patients when coadministration of food is used in the Schilling procedure (46) has first been speculated by Allen et al. (16) and it may well explain why all but a minor variable fraction of the administered Cbl was sequestered to R proteins in any one of the EPI patients studied (see Table I). More direct evidence supporting this view was obtained with the observations made with patient VI. The pH in the gastric juice of this EPI patient was approximately neutral, obviously because of admixing with the 800–1,000-ml/d (47) of the alkaline bile (44) incoming through the choledochogastroanastomosis. The Sephadex G-200 radiochromatogram obtained with the intestinal aspirates from this patient showed that no sequestration of Cbl from IF to R protein, or vice versa, had occurred during transport indicating that in EPI combined with an absence of a sufficiently acidic pH in gastric juice, the vitamin will be distributed to both IF and R proteins in molar proportions similar to the relative concentrations of these proteins in gastric juice.

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