Radioimmunoassay for Assessing Exocrine Pancreatic Insufficiency, Based on the Differential Enzymatic Degradation of Cobalamin-Binding Proteins

George Marcoullie, Jean-Louis Gueant, and Jean-Pierre Nicolas

Pancreatic proteases degrade the protein moiety of the R protein–cobalamin complex but not the intrinsic factor–cobalamin complex. Accordingly, we used these two proteins as substrates in an in vitro enzymatic assay to assess pancreatic function by incubating basal jejunal fluids with a mixture of intrinsic factor and cyanocobalamin coupled to R-type protein and then using immunoprecipitation to determine the distribution of isotope-labeled cobalamin bound to the two proteins. With normal jejunal fluids, 91.2 (SD 6.1)% and 4.5 (SD 5.5)% of cyanocobalamin was precipitated with antisera to intrinsic factor and anti-R protein, respectively. In the patients’ jejunal fluids, the cyanocobalamin precipitated with the respective antisera was 5.3 (SD 10.0)% and 96 (SD 6.2)%. In patients with other gastrointestinal problems, the sequestration of cobalamin was indistinguishable from that observed with normal fluids. The clear cut discrimination this radioimmunoassay provided between abnormal and normal samples was confirmed by parallel comparative chromatographic analysis.

Additional Keyphrases: jejunal fluid · intrinsic factor · R protein · vitamin B₁₂ · cyanocobalamin · malabsorption syndromes · gastrointestinal system · chromatography (on Sephadex) compared

The radioimmunoassay we describe here is based on the “inhibited cobalamin (Cbl) absorption theory” (1–4) and on findings obtained by in vivo studies in this laboratory (4–6). We found that under physiological conditions Cbl binds preferentially to R-type proteins in the gastric succus and that pancreatic proteases degrade this complex, with subsequent transfer of Cbl to IF (4–6). In contrast to the susceptibility of R proteins, IF is strongly resistant to the synergistic effect of pancreatic enzymes and presumably of the other proteases and glycosidases (4, 2, 4, 6) in the intestine, and it is the only one among all known Cbl binders (7–10) that, after complexing to Cbl, can attach to specific receptor sites and thereby facilitate Cbl absorption in the ileum. Indeed, the latter interaction is a prerequisite for physiological absorption of Cbl. In exocrine pancreatic insufficiency, the R protein–Cbl complexes remain undegraded because less pancreatic zymogens are secreted in the jejunal fluid (1–6). These findings have helped to define the mechanism of malabsorption of Cbl in exocrine pancreatic insufficiency as a consequence of the failure to degrade the R proteins, which are biologically inactive and inhibit Cbl absorption (4–11). Accordingly, if jejunal fluid could induce similar specific changes in IF and R proteins in vivo, presumably such specific changes could constitute the basis of a diagnostic test for the evaluation of exocrine pancreas and for the differential diagnosis of malabsorption syndromes of Cbl. Such a test would be of practical value if the changes observed during in vivo transport could be reproduced in vitro with small volumes of jejunal fluid.

In the method reported here, as little as 170 μL of jejunal fluid can, within 60 min, induce such clearcut and specific changes in a mixture of IF and R protein that a subsequent simple immunoprecipitation reaction provides useful information for assessing the exocrine pancreatic function.

Materials and Methods

Subjects

Controls. The subjects referred to here as “controls” were normal in the sense that they had no medical history of exocrine pancreatic dysfunction; showed no evidence of exocrine pancreatic insufficiency on physical examination; showed no calcifications in the upper gastrointestinal series; had neither steatorrhea nor diarrhea; and certain variables for serum and jejunal fluid pertinent to exocrine pancreatic function were found to be within normal limits.

Patients. Patients were subjects with proven exocrine pancreatic insufficiency. The diagnosis had been based primarily on a positive medical history, including chronic ethanol abuse, pancreatic calcifications, steatorrhea, ultrasonographic findings, increased fat excretion in the stool, and (or) absence or diminished pancreatic enzyme activities in the jejunal fluid (see Table I below).

Other patients. These subjects fulfilled many of the criteria described under Controls. They were, however, patients with diagnosed pathological conditions of the digestive system (Table I) that we thought might be associated with malabsorption or disturbed transport of Cbl.

Samples and Reagents

Radioactive compounds and other reagents. Cyanocobalamin (CNC) (215 Ci/g), Na⁺¹²⁵I (100 mCi/g), and tritiated water (³H₂O, 5 kCv/L) were purchased from Amer sham International, Amersham, Bucks, U.K. Bovine gamma globulin and phenylmethylsulfonyl fluoride were from Sigma Chemical Co., St. Louis, MO. Aprotinin (Iniprol) was from Laboratoire Choey, Paris, France. Crytralline Cbl, purified human serum albumin (HSA), and purified human serum albumin (HSA), and purified human
IgG were from Fluka AG, Buchs, Switzerland.

Biological fluids. We obtained informed consent from all subjects in accord with the Declaration of Helsinki, after approval by the Medical Faculty of the University of Nancy. None of the patients or the healthy subjects investigated here received a test meal or any other kind of stimulation, including food or any preparation of pancreatic extract, for at least 12 h before jejunal fluid was collected. We aspirated, on ice, approximately 10 mL of this fluid from each subject, using a 20-mL disposable syringe and a naso-gastrointestinal tube. The 150-cm, 1-mm (i.d.) tube had several side holes in its distal part (the last 3 cm); when placed approximately 130–140 cm from the incisor teeth, the tube always opened into the jejunum below the duodenojejunal flexure. After the pH of each fluid was adjusted to 8.0 by dropwise addition of 0.1 M NaOH solution, all particulate material in the fluids was removed by centrifugation.

De-pepsinised human gastric juice, serum from patients with pernicious anemia, and the antisera to R protein and to IF, raised in rabbits, were obtained or prepared as we have described (4, 6, 7). The anti-IF sera contained type II antibody that bound more than 40 pmol of IF per milliliter of serum. The anti-R protein and anti-IF antisera each were diluted with phosphate buffer (0.1 M, pH 7.4) to bind more than 30 pmol of R protein or IF per milliliter of diluted serum.

Procedures

Tests for exocrine pancreatic function. We determined the activities of chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), and lipase (EC 3.1.1.3) activities (4, 12, 13) in jejunal fluids immediately after collection. We used the Schilling procedure for measuring cobalamin absorption and measured fat in stool several days before or after the intubation, as we have described elsewhere (4, 6).

Gel chromatography. Sephadex G-200 columns, 2.5 x 100 cm, flow rate 11–13 mL/h at 4 °C, were equilibrated with phosphate buffer (0.1 M, pH 7.4) containing 154 mmol of NaCl, 0.2 g of NaHCO3, 3.5 mg of phenylmethylsulfonyl fluoride, and 5 x 105 units of aprotinin per liter. We used as marker proteins purified human IgG and HSA labeled with 125I by the Chloramine-T method (14). These marker proteins and other calibration components were included in each chromatographic sample and were eluted simultaneously with the proteins under study, 1.0- to 2.0-mL fractions being collected. We added the following markers to each sample used: 3 mg of Blue Dextran 2000 (Pharmacia), 72 ng each of 125I-labeled IgG and 125I-labeled HSA (0.3–0.5 μCi), and 1 μL of 3H2O diluted 2000-fold with 1H2O.

Radioimmunoassays and chromatographic analysis. The radioimmunoassay in its final form consisted of the following steps. Prepare 0.3 mL of a mixture containing 0.15 pmol of free IF and 1.26 pmol of CN125ICoCbl (6 nCi) precoupled to an excess of salivary R protein (4.1 pmol), in 0.1-mL aliquots, into three numbered tubes. Then add 170 μL of the intestinal fluid (from patient or control subject) to tubes 1 and 2, and adjust the final volume of each of the three tubes to 0.4 mL by adding buffer without enzyme inhibitors. After 60 min at room temperature, mix with each incubation preparation (hereafter referred to as "primary incubation mixtures") 0.6 mL of phosphate buffer (0.1 M, pH 8.0) containing 154 mmol of NaCl, 0.2 g of NaHCO3, 1 x 106 units of aprotinin, and 3.5 mg of phenylmethylsulfonyl fluoride per liter. Then incubate each incubation mixture separately as follows. Incubate the first, second, and third 1.0-mL aliquots of the primary incubation mixtures with, respectively, 0.3 mL of pernicious anemia serum (or rabbit antiserum to IF), 0.3 mL of antisemur to R protein, and 0.3 mL of buffer (these mixtures are hereinafter referred to as "secondary incubation mixtures"). Then add 0.3 mL of

### Table 1. Clinical and Laboratory Data on All Subjects Studied

<table>
<thead>
<tr>
<th>Fat in stool, g per day</th>
<th>Patients with exocrine pancreatic insufficiency</th>
<th>Other patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chymotrypsin</td>
<td>Trypsin</td>
<td>Lipase</td>
</tr>
<tr>
<td>I</td>
<td>20.43</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>8.0</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>III</td>
<td>4.1</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td>IV</td>
<td>2.5</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*We did not measure fat in the stool of most of the normal subjects because they had no evidence for or history of steatorrhea.

**Schilling test procedure without (Stage I) and with (Stage II) co-administration of intrinsic factor.

†Calculated by integration of the peaks representing IF and R protein in Sephadex G-200 chromatography.

§Missing data, owing to experimental loss or insufficient sample.

‡All had pancreatic calcifications.
bovine gamma globulin (20 g/L solution in phosphate buffer, 0.1 mol/L, pH 7.4) to each of the secondary incubation mixtures and precipitate the immunocomplexes contained in each one of the first two incubation mixtures by adding 1.6 mL of a 300 g/L solution of Na₂SO₄. Measurement of the third incubation mixture reflects the concentration of free CN⁺³⁷Co]Cbl, if any, the latter being quantified after pelleting the content of the third reaction tube with an equal volume of hemoglobin-coated charcoal.

In a second series of parallel experiments, primary incubations (see above) of jejunal fluids with the mixture of IF, R protein, and complexes of R protein–CN⁺³⁷Co]Cbl, and secondary incubation of mixtures with the anti-IF and anti-R protein sera were carried out as described above, except that we used 450 nCi instead of 5 nCi of CN⁺³⁷Co]Cbl (2.54 pmol) precoupled to R proteins. In these experiments each one of the three incubation mixtures was mixed with the molecular markers described above and eluted through separate Sephadex G-200 columns (without any treatment with Na₂SO₄ or hemoglobin-coated charcoal).

Results

Tables 1 and 2 summarize the clinical data and the measurements we obtained on using the above-described chromatographic analysis. The pH of these basal jejunal fluids correlated very poorly with the other indices of exocrine pancreatic function.

Analysis of intrinsic factor, R proteins, and cyanocobalamin before interaction with jejunal fluids. When we chromatographed through Sephadex G-200 the standard mixture of free IF and R protein coupled to CN⁺³⁷Co]Cbl, we obtained the radiochromatogram shown in Figure 1. In this instance, we applied the sample onto the column about 2.5 h after its preparation. It is evident from Figure 1A that almost all CN⁺³⁷Co]Cbl is coupled to a protein that has an estimated molecular mass of 120,000 Da. When we chromato- graphed a similar sample that was incubated with 0.3 mL of the anti-R protein serum prior to chromatography, the peak of CN⁺³⁷Co radioactivity shifted to the void volume (Figure 1B). Less than about 3% of the CN⁺³⁷Co radioactivity was eluted after HSA, apparently representing CN⁺³⁷Co]Cbl that was transferred spontaneously to the unsaturated IF contained in the sample. Conversely, we saw no significant change in the size or position of the major CN⁺³⁷Co-radioactivity peak (shown in Figure 1A) when the sample was incubated with the anti-IF serum and then filtered (Figure 1C). In this instance, the minor fraction of the CN⁺³⁷Co radioactivity that did not react with the anti-R protein serum (see Figure 1B) is now totally excluded from the gel, indicating that the unreacted minor peak of CN⁺³⁷Co]Cbl in Figure 1B was coupled to IF. It is therefore clear that the CN⁺³⁷Co]Cbl in the mixture of gastric juice and saliva was almost entirely coupled to R protein and that no appreciable spontaneous

Table 2. Ileal Absorption vs Sequestration of Cobalamin

<table>
<thead>
<tr>
<th>Schilling test: CN⁺³⁷Co]Cbl excreted into urine (% of total oral dose)</th>
<th>Sequestration of CN⁺³⁷Co]Cbl to IF by jejunal fluid (% of total) as quantified*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Stage II</td>
</tr>
<tr>
<td>Patients with exocrine pancreatic insufficiency</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8.9</td>
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<tr>
<td>II</td>
<td>28.1</td>
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<td>III</td>
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<tr>
<td>IV</td>
<td>16.6</td>
</tr>
<tr>
<td>Other patients</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12.5</td>
</tr>
<tr>
<td>II</td>
<td>33.7</td>
</tr>
<tr>
<td>III</td>
<td>17.4</td>
</tr>
<tr>
<td>IV</td>
<td>10.2</td>
</tr>
<tr>
<td>Control subjects</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23.0</td>
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<tr>
<td>II</td>
<td>24.1</td>
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<tr>
<td>III</td>
<td>33.6</td>
</tr>
<tr>
<td>IV</td>
<td>24.2</td>
</tr>
</tbody>
</table>

*Same as in Table 1.
sequestration of the vitamin to IF occurred during the standard 2-h incubation period we used for the primary and secondary reactions with jejunal fluids.

Specific interactions of normal jejunal fluid with intrinsic factor and R proteins. We incubated, and then filtered through Sephadex G-200, each one of the "control" jejunal fluids with the standard mixture of IF and CN[57Co]Cbl–R protein complex (Figure 2A). We found that an average of 96% of the CN[57Co]Cbl that was precoupled to R protein (see Figure 1) now was eluted as a well-defined, symmetrical peak, emerging after the 125I-labeled HSA marker protein. The corresponding average molecular mass of

![Diagram](image)

Fig. 2. Examples of Sephadex G-200 radiochromatograms obtained with control jejunal fluids (aspirated from normal subjects or other patients without exocrine pancreatic insufficiency) after incubation with the mixture of free IF and CN[57Co]Cbl. Solid line: precoupled to R proteins; when this incubation mixture was filtered alone (A), after incubation with anti-R protein (B), and after incubation with anti-IF sera (C). More than 97% of the CN[57Co]Cbl precoupled to R proteins (Fig. 1) now appears to be coupled to IF (Cbl-IF) or [Cbl-IF]-IgG. Symbols and markers as in Fig. 1

57 000 Da is very similar to the known molecular mass of IF measured in gel filtration (16). This peak remained unchanged in parallel experiments in which we incubated each one of the analogous primary incubation mixtures with the anti-R protein serum before gel filtration (Figure 2B). A barely-distinguishable peak of 57Co radioactivity shifted to the column void volume (see Figure 2B). The same major 57Co-reactive peak, shown in Figure 2A, shifted to the column void volume when we incubated the analogous primary incubation mixture with anti-IF serum and then filtered it through the column (Figure 2C). About 12% of the total 57Co reactivity now eluted between the 125I-labeled IgG and HSA marker proteins, indicating that after incubation with normal jejunal fluids the major fraction of CN[57Co]Cbl had been sequestered to a protein that, as judged from molecular mass and antigenic structure, is IF.

We also carried out similar series of experiments on jejunal fluids from each one of the "other patients" described in Tables 1 and 2, and found their capacity to sequester Cbl to IF to be indistinguishable from that observed with the "controls" (radiochromatograms not shown; see tabulated data in Tables 1 and 2).

Diminished sequestration of cobalamin to intrinsic factor after interaction with jejunal fluid from patients with exocrine pancreatic insufficiency. We repeated the series of three experiments shown in Figure 2, using individually the jejunal fluids aspirated from patients with exocrine pancreatic insufficiency. Figure 3 represents a typical example of the three analogous radiochromatograms and shows that (a) the major fraction, if not all, of the CN[57Co]Cbl that was initially coupled to R protein (see Figure 1) remained coupled to a protein with a molecular mass of 120 000 Da (Figure 3A), which is very similar to the known molecular mass of R proteins estimated by gel filtration (16); that (b) the major peak in Figure 3A certainly represents R protein, because in the reaction of the standard primary incubation mixture with anti-R protein serum, this peak shifted into the column void volume (Figure 3B); and that (c) a minor fraction of CN[57Co]Cbl represents immunoreactive IF, because this peak shifted to the column void volume after incubation with the anti-IF serum (Figure 3C).

Indices of exocrine pancreatic function vs sequestration of cobalamin from R proteins to intrinsic factor. We calculated the proportion of Cbl that was eluted coupled to R proteins and IF in the chromatographic analysis described above by integration of the geometrical area representing each peak (Table 1). When we used the anti-R protein serum as a probe to distinguish IF and R protein in gel filtration, an average of 88.7 (SD 16.0)% of the total CN[57Co]Cbl that initially was bound to R protein (as shown in Figure 1) remained bound to the same protein after interaction with the patients' jejunal fluid (see Figure 3B). When we used anti-IF serum to separate IF from R-proteins, a value very close to this figure of 83.6 (SD 17.2)% was calculated as the average fraction of CN[57Co]Cbl that remained bound to R proteins after incubation with the patients' jejunal fluids (see Figure 3C). Because 3.4 (SD 3.7)% of the CN[57Co]Cbl had been transferred to IF without pre-incubation with jejunal fluids (see Figure 1), we estimated the average proportion of the vitamin sequestered from R-protein to IF after interaction with the patients' jejunal fluids to be <10%. In contrast to this, the normal jejunal fluids sequestered to IF more than 96.8 (SD 7.3)% of the vitamin that was initially coupled to R proteins (see Figure 2). In retrospect, we estimated from the data shown in Table 1 that the average chymotrypsin
activity was as low as 1.6 (SD 4.4) kU/L for the patients' jejunal fluids and as high as 46.0 (SD 4.6) kU/L for all subjects without exocrine pancreatic insufficiency, and that the average trypsin and lipase activities were 0.8 (SD 0.7) and 0.3 (SD 0.4) kU/L, respectively, for patients, and 4.5 (SD 4.8) and 2.4 (SD 3.3) kU/L, respectively, for control subjects.

Similarly, using data from Table 1, we calculated that the average undigested fat in stool was 8.8 g per 24 h for the patient group. We previously reported (4) that the average undigested fat in the stool of normal subjects, similarly studied, was 2.5 g/24 h.

Ileal absorption of cobalamin vs transfer of cobalamin from R proteins to intrinsic factor. We used the Schilling test to evaluate ileal absorption of Cbl. The results (Table 1) demonstrate that normal subjects, whose jejunal fluids can transfer in vitro almost 100% of CN(57Co)Cbl from R proteins to IF (see Figure 2), exhibit, in addition, normal ileal absorption of Cbl with a mean fraction of 23.9% (SD 7.8%) of the total oral dose excreted into the urine. On the other hand, we found that the jejunal fluids from patients with exocrine pancreatic insufficiency did transfer to IF only a relatively small part, as little as 10%, of the total CN(57Co)Cbl that was precoupled to R proteins. We also found that in these patients with such "ineffective" jejunal fluids, the absorption of CN(57Co)Cbl was markedly lower than normal, with only 8.2% (SD 7.5%) of the total oral dose being excreted into the urine.

Evaluation of cobalamin sequestration by radioimmunoassay vs chromatographic analysis. Using the present radioimmunoassay, we obtained the results tabulated in Table 2. Both the radioimmunoassay and the chromatographic analysis clearly demonstrate a failure of the patients' jejunal fluids to transfer Cbl from R proteins to IF, which in turn is comparable to the ileal malabsorption of Cbl. We observed, however, that the difference in the magnitude of this sequestration between normal subjects and patients with impaired exocrine pancreatic function is consistently more significant in the radioimmunoassay measurements than in those made chromatographically (cf. Tables 1 and 2).

The slightly higher values obtained with the radioimmunoassay could be due to numerous factors. We considered the possibility that the prolonged physical separation of the immunocomplexes from the corresponding unreacted antibodies and antigens (IF or R proteins) during gel filtration or salting out can result in a dissociation of immunocomplexes to maintain the equilibrium of the reaction according to the law of mass action. We also considered that such a dissociation would be expected to be greater during the 48-h gel-filtration separation than during the 30-min salting-out procedure used in radioimmunoassay. This in turn would result in falsely decreased estimations of concentration of immunocomplexes containing either R protein or IF when the immunocomplexes are quantified chromatographically. If this were the case, then some free (not coupled to immunocomplexes) IF and some free R protein ought to be present, respectively, in the peaks representing R protein and IF in the Figures 2C and 3C, and 2B and 3B, respectively. Accordingly, to examine this possibility, we obtained samples from the fractions corresponding to each of the 57Co-radioactivity peaks in the included column volumes of the radiochromatograms shown in Figures 2B, 2C, 3B, and 3C, and assayed them for their relative content of free R protein and free IF. We carried out these assays as described under Radioimmunoassays and chromatographic analysis, using immunoprecipitation with Na2SO4, except that we used no antiserum. These assays showed that free R protein and free IF indeed constituted respectively 8 to 10% and 9 to 14% of the 57Co radioactivities in the peaks, which we deemed to represent, respectively, immunocomplexes of IF and R protein only.

Simple quantification of cobalamin liberated from R proteins is an unreliable index of exocrine pancreatic function. Since the beginning of this study, we have considered the possibility that a direct measurement of Cbl liberated from R proteins in the presence of jejunal fluid might correlate with the rate of degradation of R proteins. This, in turn,
would suffice to evaluate the exocrine pancreatic function. In this instance we could use simple adsorption of free Cbl to charcoal to quantify the magnitude of degradation of R proteins. We would thus obviate the use of anti-IF or anti-R protein sera required in the radioimmunoassay. Accordingly, we investigated this possibility by incubating jejunal fluids from normal subjects with CN\(^{57}\)CoCbl pre-coupled to R proteins alone and then assaying aliquots of these samples for the presence of free Cbl, using charcoal (16). We also filtered aliquots from the same incubation samples through Sephadex G-200. We found, using the charcoal assay, that the fraction of Cbl that appeared to be freed constituted only 10 to 20% of the total CN\(^{57}\)CoCbl in the sample. This is clearly illustrated in Figure 4A, which was obtained from the column void volume (Figure 4B) and the result eluted as in the case where no incubation with anti-serum was used (Figure 4A).

**Discussion**

We have now duplicated in vitro our previous observations about the in vivo interactions of jejunal fluid with Cbl, R proteins, and IF (see the *Introduction*). These observations, as discussed below, provide the basis for a test with the potential to evaluate the exocrine pancreatic function, and they show that the differential interactions of the R protein–Cbl complex with normal and patients' jejunal fluids evidently also occur under basal conditions.

As indicated in the introduction of this paper, in vivo observations (4–6) that normal—but not patients’—jejunal fluids can rapidly sequester Cbl from R proteins to IF have encouraged us to explore this phenomenon in vitro. Similarly, the need to obviate the many disadvantages and limitations of certain tests for exocrine pancreatic function that have evolved over the past 40 years (reviewed in 17) prompted us to develop the present assay. The need for a practical, fast, and sensitive test for exocrine pancreatic function becomes clear from the simple epidemiological data that now are available in the United States: (a) the crude death-rate from chronic pancreatitis is 0.1 to 0.3 per 100,000; (b) pancreatic malignant neoplasms account for 2 to 4% of all cancers; (c) cystic fibrosis is the most frequent genetic disease (17); and (d) numerous unpredictable factors or co-existing disease other than the actual pancreatic zymogen output (e.g., concentration of bicarbonate in plasma, the presence of gastric juice and bile in the duodenum, bacterial overgrowth, etc.) can modify the apparent magnitude that is measured as exocrine pancreatic function by several of the currently available tests.

Measurements made with the present radioimmunoassay system allow a preliminary appraisal of the diagnostic value of this test in different clinical settings: the transfer of Cbl from R proteins to IF, as measured by the radioimmunoassay, provides a new and highly discriminant value between abnormal and normal jejunal fluids. Furthermore, by using reactions with specific antisera to IF and to R protein and gel filtration analysis, we confirmed the results obtained with the radioimmunoassay and characterized more specifically the reactions that led to those results. Thus we concluded from the radiochromatograms shown in Figures 2 and 3 and the data in Table 2 that as much as 95% of Cbl is sequestered to IF with normal jejunal fluids, but as little as 10% with patients' jejunal fluids. The normal sequestration of Cbl observed with jejunal fluid aspirated from patients with various gastrointestinal diseases (bacterial overgrowth, duodenal ulcer under cimetidine administration, achlorhydria, hepatic cirrhosis, mesenteric arteritis, diarrhea, atrophic gastritis, etc.) indicates that this test may be highly specific and that the sequestration is directly related to exocrine pancreatic function. Schilling test values may be abnormal and undigested fat or chymotrypsin may be present in the stool in pathological states such as bacterial overgrowth, mesenteric arteritis, and achlorhydria.

As indicated in the *Results* section, the quantitative measurements of IF and R proteins in the secondary incubation mixtures that we prepared with antisera to IF and R protein, respectively, were both higher when we used the radioimmunoassay procedure instead of the chromatographic analysis. This apparent discrepancy can now be explained according to the principles of the law of mass action, as shown in Figure 4: in gel filtration the presence of 8 to 10% and 9 to 14% free R protein and free IF in the respective

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peaks that initially were deemed to represent solely immunocomplexes demonstrates that a minor fraction of the immunocomplexes does spontaneously dissociate when the latter formations are physically separated from the excess free antibody and free antigen (that is, IF or R protein). Conversely, this dissociation is presumably negligibly small when the preformed immunocomplexes are precipitated rapidly by salting-out.

We have been aware of the fact that pancreatic proteolytic enzymes might degrade the antibodies used in the assay as they did the R proteins. For this reason all incubations involving antibodies have been carried out in the presence of the enzyme inhibitors aprotinin and phenylmethylsulfonyl fluoride. As shown in Figures 2 and 3, the antisera to IF and R protein have been used in sufficient quantities to couple all IF or R proteins present in the incubation mixtures; thus the pancreatic proteases were inhibited and the antibodies remained intact and reactive throughout the assay.

As we can see from the results shown in Tables 1 and 2, the failure of the intestinal fluids to degrade the R protein–Cbl complex was significantly greater in patients with exocrine pancreatic insufficiency. This was associated with the clinical Cbl malabsorption, as measured by the Schilling test (23.9% urinary exception in normal subjects, 8.2% in patients), with the fecal fat test parameters, and with low values for chymotrypsin and lipase in the intestinal juice. It is therefore clear that both the diminished pancreatic proteolytic activity in jejunal fluid and the failure to digest fat parallel the poor capacity of the jejunal fluid to sequester Cbl from R protein to IF in vitro. Conversely, normal pancreatic proteolytic activity in jejunal fluid and also normal digestion of fat are associated with an enormous potential of the corresponding jejunal fluid (that is from subjects without exocrine pancreatic insufficiency) to transfer Cbl from R proteins to IF. In these clinical correlations, we did not calculate any statistical coefficients because of the relatively small number of patients studied; it has been extremely difficult to recruit a greater number of patients with exocrine pancreatic insufficiency. Notwithstanding the large number of patients with this problem in our hospital, we found it relatively convenient to recruit normal subjects or other patients rather than exocrine pancreatic insufficiency patients in this study.

We attempted to obviate the use of antisera to IF or R protein in this test by directly measuring the CNI\(^{57}\)CoCbl freed as R proteins were being degraded. In this instance, simple adsorption of free CNI\(^{57}\)CoCbl onto charcoal might provide an indirect measure of the pancreatic enzyme activity. However, as we can see from the radiochromatograms of Figure 4, even though incubation with normal fluid can degrade R proteins, the vitamin is being only partly sequestered to endogenous IF (contained in the basal jejunal fluid in relatively low concentrations (5)). The remaining major fraction of the vitamin was bound to another, low-affinity Cbl-binder, from which Cbl was spontaneously dissociated and which is not immunoreactive IF. The latter low-affinity binder may represent a derivative of R-type proteins invariably present in jejunal fluids (named “corrinoid binder”; see ref. 16) or a degradation product derived from the CNI\(^{57}\)CoCbl–R protein complex (2). Clearly, whatever the identity of the latter molecule, the vitamin thus liberated from R proteins will still appear to be bound to other R proteins as well as to the free endogenous IF that is present in most jejunal fluids. This, in turn, may obfuscate the actual magnitude of the enzymatic effect on R proteins if the charcoal-adsorption technique is used.

Despite the fact that this radioimmunoassay clearly distinguishes jejunal fluids of patients with exocrine pancreatic insufficiency from those of normal subjects and of some other patients with various gastrointestinal diseases, it still remains to be shown if this test can discriminate to the same extent from the entire array of diseases associated with dysfunctions of the alimentary tract. In theory, however, and also in view of the principles on which this test has been based (“inhibited cobalamin absorption theory”), there is no obvious reason to speculate a discrepancy in the normal in vitro transfer of Cbl from R proteins to IF in any known pathological state in the intestine other than pancreatic dysfunction. For these reasons, and also because of the findings presented here, we believe that the present radioimmunoassay system will be a useful and practical diagnostic tool in the routine assessment of exocrine pancreas and in the differential diagnosis of intestinal malabsorption of Cbl. The small volume (170 \(\mu\)L) of jejunal fluid required for the present test is conveniently obtained. A small aliquot (a few hundred microliters) of jejunal fluid can be aspirated before breakfast by use of a single naso-gastro-jejunal tube, 1-mm in diameter, or during diagnostic endoscopy without prior direct or indirect stimulation of the pancreas. Furthermore, the test can be completed, including the radioimmunoassay step, within 2.5 to 3 h. The test does not require hospitalization of the patient or administration of radioisotopic compounds or of other biological substances (such as heterologous or homologous IF, cobamimide, R protein) or injection of stimulants such as hormones (17, 19, 20). It does not require collection of either a 24-h urine specimen (Schilling test) or 72-h stool (fecal fat, nitrogen and chymotrypsin) or 80-min continuous collection of intestinal fluid (bicarbonate output)—and so forth.

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References