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Pure Human Pancreatic Juice Directly Enhances Uptake of Cobalamin by Guinea Pig Ileum *in Vivo* (41996)

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Abstract. Although pancreatic enzymes clearly degrade R binder, a nonintrinsic factor binder, the full scope of the pancreatic role in cobalamin absorption remains the subject of debate. Therefore the direct effect of pure human pancreatic juice (PPJ) on ileal cobalamin absorption in the absence of intrinsic factor was studied. PPJ significantly enhanced cobalamin uptake in guinea pig ileal loop perfused *in vivo*. It did not do so in the jejunum. This PPJ activity in the ileum was further stimulated by enteropeptidase and inhibited by aprotinin. The intestinal mucosa remained intact during our study by morphologic and inulin clearance criteria and behaved normally with respect to intrinsic factor and nonintrinsic factor binders. Since no intrinsic factor was present in the perfusate, PPJ must directly enhance cobalamin uptake by the ileum, perhaps promoting cobalamin attachment to receptor sites for subsequent transport by intrinsic factor. PPJ thus seems to affect cobalamin absorption at several levels. Previous studies have established its interaction with luminal R binders and with bile. The findings now indicate that pancreatic juice may have an additional, more direct role in promoting cobalamin absorption in the ileum.

Patients with pancreatic insufficiency often have abnormal cobalamin (vitamin B_{12}) absorption (1), but the mechanism has not been fully clarified. As reviewed elsewhere (2, 3), pancreatic action does not appear to affect intrinsic factor directly. Considerable evidence (4, 5) supports the proposal by Okuda *et al.* (6) that pancreatic enzymes prevent the sequestration of cobalamin by gastrointestinal nonintrinsic factor binders. Such pancreatic action would allow cobalamin to be bound by intrinsic factor instead and be absorbed. Pancreatic proteases in the small intestine clearly degrade the nonintrinsic factor "R binders" which bind cobalamin initially in the gastric lumen (4, 5, 7, 8). Nevertheless, disagreement persists regarding the precise mechanisms of the interrelationship between pancreatic exocrine secretions, R binders and intrinsic factor, and other pancreatic roles have been suggested (9–11).

Much of the difficulty in unraveling the chain of events within the lumen stems from the lumen's relative inaccessibility. The complex interactions of the many secretions with their different cobalamin binders, enzymes, and variable fluxes of the vitamin compound the difficulty. The potential for technical artifacts is further complicated by the variability of animal and human models. In the dog, for example, pancreatic juice was reported to actually contain intrinsic factor (12, 13), which has not been observed in other animals. Rats have been frequently used to study pancreatic effect, yet paradoxically they do not always develop cobalamin malabsorption after pancreatectomy (14). Moreover, pancreatic extracts, particularly in high doses, appeared to actually inhibit cobalamin absorption in some animal and human studies (14–18). Such effect was not seen with more physiological concentrations (14, 15, 18). High concentrations may even damage the gut in subtle ways (18).

To simplify at least one variable, we decided to study pure human pancreatic juice (PPJ). Previous studies had used animal pancreatic juice, crude enzyme extracts, or human duodenal juice. The studies to be described extend our previous observations with PPJ (19). They examine the direct action of PPJ in the ileal phase of cobalamin absorption unaffected by intrinsic factor and R binder. A preliminary report of these data has appeared in abstract form (20).

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Materials and Methods. PPJ that behaved normally *in vitro* had been previously obtained following stimulation with cholecystokinin–pancreozymin of a healthy volunteer and three patients with chronic pancreatitis but without pancreatic insufficiency. The criteria for these diagnoses and the characterizations of these four specimens have been published (19). The specimens were collected during retrograde cannulation of the pancreatic duct under endoscopic visualization (21, 22). They were stored at −70°C until used in our studies. Subsequent storage was in small aliquots at −20°C. All four specimens were clear and colorless, and gave normal assay results for protein, zymogen, and enzymes. They degraded human R binder normally *in vitro* (the specimens displayed protease activity even without addition of enteropeptidase) and had negligible cobalamin-binding capacity and cobalamin content (19). The cobalamin absorptive status of the four subjects was presumed to be normal but was not tested directly.

Normal gastric juice was obtained from a control subject; intrinsic factor constituted 78% of its cobalamin-binding capacity by immunoassay (23). Gastric juice lacking intrinsic factor was aspirated from a patient with classical pernicious anemia. These juices were depepsinized, neutralized, and stored at −20°C. Radioactive cyanocobalamin ($^{57}CoB_{12}$) of 220 μCi/μg sp act was purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. Human enteropeptidase, partially purified by Sephadex gel chromatography and free of trypsin bound to α_2-macroglobulin, was a gift from Dr. H. Rinderknecht, Veterans Administration Hospital, Sepulveda, California, and was stored at 4°C; its concentration was 11 mIU/ml. One milliliter was incubated with the 0.1-ml aliquot of PPJ for 2 hr at room temperature before perfusion. Aprotinin (bovine lung trypsin inhibitor) was purchased from Sigma Chemical Company, St. Louis, Missouri. It was diluted to a concentration of 11 kallikrein inhibitor units/ml, and 1 ml was incubated with 0.1 ml of PPJ for 30 min at room temperature before perfusion.

Male HLA Hartley guinea pigs weighing 200–500 g (Simonsen Labs, Inc., Gilroy, Calif.) were allowed free access to water and food but fasted for 12 hr preceding the experiment. Each animal was the subject of a single experiment. Following ethyl ether anesthesia, the small bowel was exposed. An inflow Tygon tube (2-mm o.d.) was inserted into the small bowel 32 cm proximal to the ileocecal valve and secured by encircling ligatures. Endogenous gastric, pancreatic, and biliary secretions thus could not enter the perfused segment. The lumen of the distal ileum, 2 cm proximal to the ileocecal valve, was cannulated with an L-shaped glass cannula. The abdominal cavity was then closed. The guinea pig was allowed to awaken and was placed in a Plexiglas restraining cage. In two control animals, "jejunal" loops proximal to the ileal region used in the other animals were cannulated for perfusion in a similar manner.

The guinea pig's body temperature was monitored with a rectal temperature probe and maintained at 37°C with a thermostatically controlled forced-air heating device. A syringe pump (H351, Sage Instruments, Cambridge, Mass.) was used to infuse normal saline solution (Cutter Medical, Berkeley, Calif.) at a constant rate of 1 ml/min for 1 hr, which reduced cobalamin-binding capacity in the perfused intestinal loop to negligible levels. No intrinsic factor could be demonstrated in the perfusate following the 1-hr wash. At the conclusion of the wash, the segment was cleared of saline and the inflow tube was connected to plastic tubing on a Buchler (Fort Lee, N.J.) Polystaltic Pump. The perfusate to be tested was placed on a magnetic stirrer under the glass cannula. The tubing from the Buchler pump was placed in the perfusate, thus creating recirculating perfusion. The pump flow rate was set at 1 ml/min. The 0.1 *M* sodium phosphate, pH 7.4, perfusate (25 ml) contained 450 pg $^{57}CoB_{12}$ and, where appropriate, 0.1 ml of PPJ or gastric juice which had been incubated with the $^{57}CoB_{12}$ before perfusion. We demonstrated in preliminary experiments that PPJ did not cause $^{57}CoB_{12}$ adherence to either cannula or tubing.

Duplicate aliquots of 0.5 ml of the perfusate were collected for counting every 30 min for 3 hr. These aliquots demonstrated that uptake was linear with time. The recirculation perfusion was then discontinued and the

bowel segment was flushed with the buffer at a rate of 1 ml/min for 15 min. Samples were counted for radioactivity in a well-type gamma counter. Initial and final counts of the perfusate were compared to a $^{57}CoB_{12}$ standard, and percentage clearance was calculated for the 3-hr period. Results were analyzed statistically using Student's t test.

[^{14}C]Inulin (220 μCi/μg, New England Nuclear, Boston, Mass.) absorption (24) was tested in several animals, using our perfusion system with and without added PPJ in order to rule out mucosal disruption or changes in water absorption caused by PPJ. The cobalamin-binding capacity of perfusate was also determined in separate $^{57}CoB_{12}$-free perfusion experiments done with and without added PPJ and enteropeptidase. Aliquots of 0.2 ml of each type of perfusate were incubated *in vitro* with $^{57}CoB_{12}$ and assayed for binding capacity by Sephadex G-200 gel chromatography using 0.1 *M* Tris–1 *M* NaCl, pH 8.6. Filtration was at room temperature using 0.9 × 60-cm columns; 15-drop fractions were collected by gravity. In additional matched experiments, the aliquots from the control perfusions were also incubated *in vitro* with 30 ul PPJ and enteropeptidase before such chromatography.

The *in vivo* effect of PPJ on luminal cobalamin-binding proteins was examined in $^{57}CoB_{12}$-free ileal perfusions. Aliquots removed after 3 min and at the end of the 3-hr perfusion were stored at −20°C. They were thawed once for simultaneous protein assay (25) and incubation of 0.4-ml portions with excess $^{57}CoB_{12}$ for 1 hr followed by fractionation on Sephadex G-200 gel as above. Elution patterns were compared to those of standard human cobalamin-binding proteins. Incubation with specific rabbit antisera, using normal rabbit serum as a control, allowed immunologic identification; shift of the binder to void volume on subsequent chromatography indicated a positive reaction. The antisera had been prepared against human R binder (using saliva as the antigen source) or intrinsic factor (using gastric juice from a patient with hereditary R binder deficiency). Antiserum to human transcobalamin II was a gift from Dr. Charles A. Hall, Veterans Administration Hospital, Albany, New York. All three antisera were shown to be specific for their respective cobalamin-binding proteins and not to cross-react with the other binder antigens, although the anti-intrinsic factor antiserum required prior adsorption with saliva R binder (26).

Results. Table I demonstrates that the ileal loop uptake of $^{57}CoB_{12}$ behaved in the normal, expected fashion. Normal gastric juice significantly enhanced uptake compared with free $^{57}CoB_{12}$ ($t = 6.97$, $P < 0.001$), whereas pernicious anemia gastric juice inhibited uptake ($t = 2.97$, $P < 0.002$). The enhancement by normal gastric juice could be eliminated by lowering the perfusion pH to 4.5 (11.3% in the single experiment).

PPJ enhanced $^{57}CoB_{12}$ uptake by ileal loop (Table I). All four PPJ specimens, tested in eight experiments, behaved similarly. PPJ-mediated uptake was nearly twice that of free $^{57}CoB_{12}$ by itself ($t = 3.46$, $P < 0.01$), though it was not as great as with normal gastric juice ($t = 3.49$, $P < 0.005$).

In striking contrast to the ileal pattern, neither normal gastric juice (2.6% ± 1.7, $n = 3$) nor PPJ (1.8% mean in two experiments) promoted uptake of $^{57}CoB_{12}$ in jejunal loops. Nor did PPJ enhance $^{57}CoB_{12}$ uptake by nonintestinal cells, exemplified by reticulocytes tested according to the method of Retief *et al.* (27) (data not shown).

Several experiments demonstrated that the PPJ enhancement effect within the ileum derived from its proteolytic activity. Preincubation of PPJ with enteropeptidase enhanced its ability to promote $^{57}CoB_{12}$ uptake even further, presumably by activating zymogens, whereas preincubation with the pro-

TABLE I. EFFECT OF PPJ AND GASTRIC JUICE ON $^{57}CoB_{12}$ UPTAKE BY GUINEA PIG ILEUM

Additive to $^{57}CoB_{12}$ in perfusate	n[a]	Percentage uptake[b]
None	7	13.8 ± 1.9
Normal gastric juice (intrinsic factor)	8	34.3 ± 2.1
Pernicious anemia gastric juice	3	3.8 ± 1.9
PPJ	8	23.9 ± 2.1
Boiled PPJ	4	12.3 ± 3.2
PPJ + enteropeptidase	4	32.2 ± 5.3
PPJ + aprotinin	4	9.8 ± 0.1

[a] n = Number of animal experiments.
[b] Mean ± SE.

tease inhibitor, aprotinin, or boiling the PPJ eliminated PPJ effect (Table I). With aprotinin or boiled PPJ, uptake resembled that of free $^{57}CoB_{12}$ itself.

PPJ activity in the ileum declined upon reuse of the PPJ stored at −20°C. In two duplicate experiments with one PPJ specimen, mean uptake of $^{57}CoB_{12}$ declined from 30 to 9.7% in 3 weeks. Uptake with a second specimen declined from 24 to 13.5%. Even after such decline, however, enteropeptidase could restore the PPJ enhancement to 22% in the latter case. Enteropeptidase by itself did not promote uptake of free $^{57}CoB_{12}$.

Studies with [^{14}C]inulin, a nonabsorbable marker (24), demonstrated no difference in water transport between control perfusions and perfusions with PPJ. Moreover, sections from ileal loops perfused with and without PPJ demonstrated no morphological abnormalities by light microscopy done at the end of the experiment. This indicates that PPJ had no measurable effect on the viability or function of the ileal loop.

A small amount of leakage of protein from the bowel into the lumen occurred over the 3-hr course of the perfusion in six animals (mean ± SD = 275 ± 246 μg/ml at the beginning vs 773 ± 378 μg/ml at the end of the perfusion). Guinea pigs perfused with PPJ behaved like the control animals in this respect. Because of this minor protein leakage and because we wanted to characterize the binders in our perfusion system, perfusate (free of $^{57}CoB_{12}$) from the six guinea pigs was also analyzed for cobalamin-binding proteins by Sephadex G-200 gel chromatography. Figure 1 illustrates a representative result. Virtually no binding was present at the beginning of perfusion (3 min); at the end of perfusion a 35,000-mol wt binding peak with a cobalamin-binding capacity of 78 pg/ml of perfusate was clearly evident. A small "shoulder" of about 60,000 mol wt (eluate fraction 33) of at most a few picograms per milliliter and a small void-volume peak may also have been present. In the other two PPJ-free perfusion experiments 35,000-mol wt peaks of 44 and 93 pg/ml binding capacity were identified. Immunologic testing revealed that this 35,000-mol wt binder was transcobalamin II, which is the predominant cobalamin-binding protein in guinea pig plasma. It cross-reacted

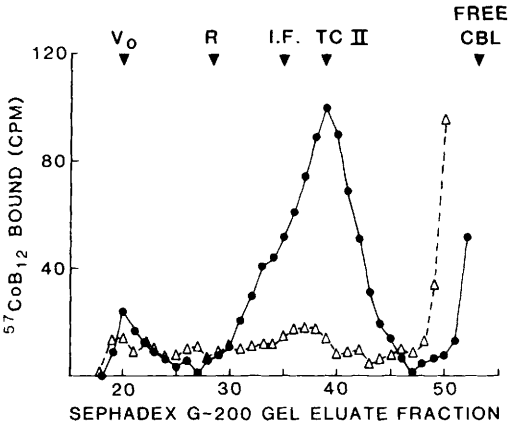

FIG. 1. $^{57}CoB_{12}$ pattern of guinea pig ileal perfusate. Negligible binding is seen in the 3-min specimen (△) in comparison with the binding at the end of the 3-hr perfusion (●). The markers are V_0 (void volume), R (human R binder or transcobalamin I, 120,000 mol wt), IF (human intrinsic factor, 55,000 mol wt), TC II (human transcobalamin II, 30,000 mol wt), and free Cbl (cobalamin, 1355 mol wt).

with our anti-human transcobalamin II antiserum but did not react at all with either anti-intrinsic factor or anti-R binder antiserum. This binder was not altered by *in vitro* incubation with PPJ activated with enteropeptidase, nor was its cobalamin-binding capacity diminished thereby.

A binder pattern similar to the one in Fig. 1 occurred in two experiments in which PPJ and enteropeptidase (without $^{57}CoB_{12}$) had been added *in vivo* to the perfusate. At the end of these perfusions, the major binding peak was also in the 35,000-mol wt region (63 and 110 pg/ml binding capacity in the two cases) with lesser 60,000-mol wt and void-volume peaks. A third perfusion was done with PPJ and enteropeptidase preincubated with aprotinin. Its subsequent gel filtration revealed a pattern unchanged from the other perfusates.

Thus, the inflow of cobalamin-binding protein, as well as that of protein generally, into the gut lumen was the same whether PPJ was present or not. It was indistinguishable among the three control animals, the two perfused with PPJ preincubated with enteropeptidase and the one perfused with PPJ preincubated with aprotinin. This inflow phenomenon, therefore, does not account for

the differences in $^{57}CoB_{12}$ uptake that we observed in the perfusion studies.

Although the small 60,000-mol wt shoulder in Fig. 1 (fraction 33) corresponds to the molecular weight of guinea pig intrinsic factor, no reaction with rabbit anti-human intrinsic factor antiserum occurred with such perfusate from either a control or a PPJ-perfused animal. In contrast, guinea pig gastric juice contained only binder which reacted fully with our anti-human intrinsic factor antiserum.

Discussion. PPJ clearly enhanced ileal cobalamin uptake in these studies. This finding fits with the widely accepted thesis that the pancreas plays an enhancing role in cobalamin absorption in man. However, it suggests a new additional mechanism since the effect was independent of R binder and intrinsic factor.

Past observations on such a direct effect of pancreatic secretion have been contradictory. Okuda (28) noted that a pancreatic enzyme preparation enhanced cobalamin absorption in the rat in the absence of intrinsic factor, but others found no such effect using human duodenal juice (29) or rat pancreatic juice (30). Our approach, therefore, was to use PPJ in studying the pancreatic role, and avoid the uncertainties inherent in using crude enzyme preparations, animal secretions, or mixed secretions such as duodenal juice. In addition, we selected lower volumes of pancreatic juice, and used an isolated intact ileal loop *in vivo* rather than gut homogenate *in vitro.*

The ileal loop model behaved normally. Intrinsic factor (normal gastric juice) enhanced cobalamin uptake by the ileum as expected, whereas such uptake was not enhanced when tested in the jejunum. Pernicious anemia gastric juice, containing only nonintrinsic factor binders, not only failed to promote ileal uptake of cobalamin but actually inhibited it compared with buffer controls; similar inhibition has been regularly demonstrated in the past in human (31), guinea pig (32), and hamster (33) gut preparations. The PPJ activity that we discovered here could not be attributed to intestinal damage noted in studies using large amounts of pancreatic materials (18). We used small amounts of PPJ. Moreover, we found the ileum to be normal on light microscopic examination, and showed with well-defined techniques employing the nonabsorbable marker, inulin, (24) that PPJ produced no changes in water absorption. Although some protein entered the lumen in the course of the experiments, this minimal secretion was the same in control and PPJ perfusions. Finally, the fact that PPJ enhanced cobalamin uptake only in the ileum, but not in the jejunum, further speaks against our results being explainable by artifactual damage to intestinal mucosa.

The only previous study of human PPJ was of a single specimen from a patient with pancreatic cancer (30). That specimen had increased the cobalamin uptake by guinea pig gut homogenate only slightly. Since that PPJ contained no trypsin activity (30), such an insignificant or negative result is not surprising. Our experiments suggest that the enhancing effect of PPJ depends on its proteolytic activity. PPJ effect was increased by enteropeptidase and inhibited by aprotinin, suggesting that trypsin was responsible for this PPJ activity. The activity also seemed somewhat labile to refreezing and thawing. The lability contrasts with the stability of the PPJ's proteolytic capacity to degrade R binder *in vitro,* which in fact tended to increase with similar handling (19). Whether this disparity between the two pancreatic activities indicates that different PPJ components were responsible for each or reflects a technical difference is not clear.

Several important questions must be addressed in interpreting and applying our findings. First, it must be noted that, although we used pure human secretions, they were tested in a heterologous system. The latter has been a problem even in the many previous studies not using human PPJ. We selected guinea pig ileum because, unlike rat intestine, it is an excellent receptor for human intrinsic factor, and has been the preferred animal model for studying cobalamin absorption with human secretions (see Ref. (34) for review). Moreover, the guinea pig more nearly resembles man by absorbing cobalamin in its distal ileum, unlike the rat. In addition, the guinea pig has no R binder in the gastrointestinal tract (35–37). Against these advantages must be weighed the fact that the

pancreatic role in the guinea pig's cobalamin absorption is unknown. Yet the usual model for pancreatic studies, the rat, may not be preferable in this respect. For example, some investigators have suggested that the rat may paradoxically fail to malabsorb cobalamin after pancreatectomy (14). The definitive study of human absorption obviously requires testing pure human secretions in human intestine. However, gut homogenate studies with PPJ are subject to artifact (R. Carmel, unpublished observations), and the technical and ethical difficulties of performing in man the studies described here are formidable. Our data thus must stand as intriguing observations whose full extrapolation to man will require confirmation.

A second question is whether any cobalamin-binding proteins could have participated in the phenomenon observed. In view of the well-established activity of pancreatic enzymes against R binder (4, 5, 7, 8, 19), we must consider whether our findings are explicable in relation to R binder activity. The PPJ contained only small amounts of R binder (19) and no R binder was identified in the perfusate. The PPJ effect could not be attributed to residual intrinsic factor in the lumen either. Intrinsic factor was washed away before perfusion, could gain no access to the closed loop, and indeed was not demonstrable immunologically in the perfusate. Even if the small "shoulder" of 60,000-mol wt binder in the final perfusate in some of our experiments had represented intrinsic factor below the limits of immunologic detection, it would not explain the PPJ enhancement of cobalamin uptake because this shoulder was also noted in control animals who were perfused without PPJ and did not have enhanced uptake.

Some transcobalamin II-like binder did enter the gut in the course of the experiments. It is not clear whether this represented a physiological process or was merely a result of the perfusion. However, PPJ, whether present *in vivo* during perfusion or *in vitro,* neither affected the amount of this binder entering the lumen nor altered its binding capacity or chromatographic characteristics. The PPJ effect, thus, seems independent of any cobalamin-binding proteins in the lumen.

The question most pertinent to man that our findings raise is why then do patients with the gastric disorder characteristic of pernicious anemia ever become cobalamin depleted. If PPJ can independently promote cobalamin absorption, the simple lack of intrinsic factor should not produce significant cobalamin malabsorption. This seeming discrepancy is best explainable by the pancreas simply playing a facilitating role here. We believe it most likely that PPJ acts by enhancing cobalamin attachment to the intestinal wall; subsequent transport of cobalamin into the cell would still require the intervention of intrinsic factor. This hypothesis is particularly strengthened by noting that the PPJ effect was specific for the ileum.

Our PPJ findings indicate that pancreatic action in cobalamin absorption is a complex process operating on several levels. Several laboratories have shown that pancreatic juice is active against human gastric R binders carrying ingested cobalamin (4, 5, 8). Our previous observations demonstrated that pancreatic juice is also active against human biliary R binders, which facilitates enterohepatic recycling of cobalamin (19). We have also suggested a role for pancreatic juice in degrading apo-R binders that do not carry any cobalamin in the human intestine (19). Others have reported an additional pancreatic action inhibiting biliary effect on gastric juice (11). Our data now indicate that pancreatic juice may have still another important and more direct role in promoting cobalamin absorption in the ileum. Although such a proposition is new and requires integration into our concepts of cobalamin absorption it is not entirely without precedent. Studies of baboons with gastrectomy have shown that secretions obtained by biliary duct cannulation can enhance cobalamin absorption in the absence of intrinsic factor (38). Direct studies in man are now required to clarify the nature and significance of these phenomena.

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