Trypsin-Like Nature of the Pancreatic Factor That Corrects Vitamin B_{12} Malabsorption Associated with Pancreatic Dysfunction

PHILLIP P. TOSKES, JULIUS J. DEREN, and MARCEL E. CONRAD with the technical assistance of GEORGE W. SMITH

From the Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20012, and the Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT Hog pancreas was subfractionated and assessed for its ability to correct vitamin B_{12} malabsorption in patients with pancreatic dysfunction and in rats with partial pancreatic extirpation. The constituent obtained from the pancreas that increased vitamin B_{12} absorption in both humans and rats was soluble at 50,000 *g*, heat labile, acid stable, and approximately 20,000–25,0000 in molecular weight. The active subfractions contained tryptic and chymotryptic but no amylase or lipase activity. Thrice-crystallized trypsin corrected the vitamin B_{12} malabsorption in both patients with pancreatic insufficiency and in rats with subtotal pancreatectomy. These data indicate that pancreatic proteolytic enzymes—in particular, trypsin—are necessary for optimal vitamin B_{12} absorption.

INTRODUCTION

Vitamin B_{12} malabsorption occurs in some patients with chronic pancreatic exocrine insufficiency (1–3) and in rats subjected to partial pancreatectomy (4). Although hog pancreatic extract corrects the absorptive defect in both humans and animals (1, 2, 4), the precise constituent in the pancreatic extract responsible for the enhancement of vitamin B_{12} absorption has not been determined.

The present study (*a*) evaluates the ability of subfractions of hog pancreatic extract to correct vitamin B_{12} malabsorption in patients with pancreatic insufficiency and in partially pancreatectomized rats, (*b*) correlates the capacity of pancreatic subfractions to correct vitamin B_{12} malabsorption with the concentrations of various pancreatic enzymes in the subfractions, and (*c*) demonstrates that crystalline trypsin possesses the capacity to enhance the vitamin B_{12} malabsorption associated with pancreatic dysfunction.

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METHODS

Vitamin B_{12} absorption studies in human subjects. Absorption was measured by the standard urinary excretion test using 0.5 μg of ^{57}Co-labeled vitamin B_{12} (1 μCi/μg) (5). Urine was collected for 24 h, counted in a gamma spectrometer with appropriate correction for geometric variation, and the results expressed as percentage of the dose excreted. In some studies various pancreatic subfractions or trypsin[1] were administered concomitantly with labeled vitamin B_{12}.

Vitamin B_{12} absorption studies in rats. Partial pancreatectomies were performed on male albino rats[2] as described by Scow (6) and modified by Toskes and Deren (4).[3] After a suitable recovery period, 1 ml of ^{57}Co-labeled vitamin B_{12} containing 5 ng of vitamin B_{12} (13–15 μCi/μg) was administered via gastric tube to fasted rats. Immediately after dosing and again 6 days later, whole body radioactivity was measured in a small animal liquid scintillation detector.[4] Percent absorption was calculated as the ratio of the net radioactivity on day 6 divided by the net radioactivity on day 1 times 100. Pancreatic subfractions and trypsin were administered with labeled vitamin B_{12} to certain animals.

Preparation of pancreatic subfractions. 9.6 g of hog pancreatic extract (Viokase[5] or Cotazyme[6]) were homogenized

[1] Worthington Biochem Corp., Freehold, N. J.

[2] Charles River CD Rats, Charles River Breeding Laboratories, Brookline, Mass.

[3] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

[4] Packard Instrument Corp., Downers Grove, Ill.

[5] VioBin Corp., Monticello, Ill.

[6] Organon Lab Ltd., Crown House, England.

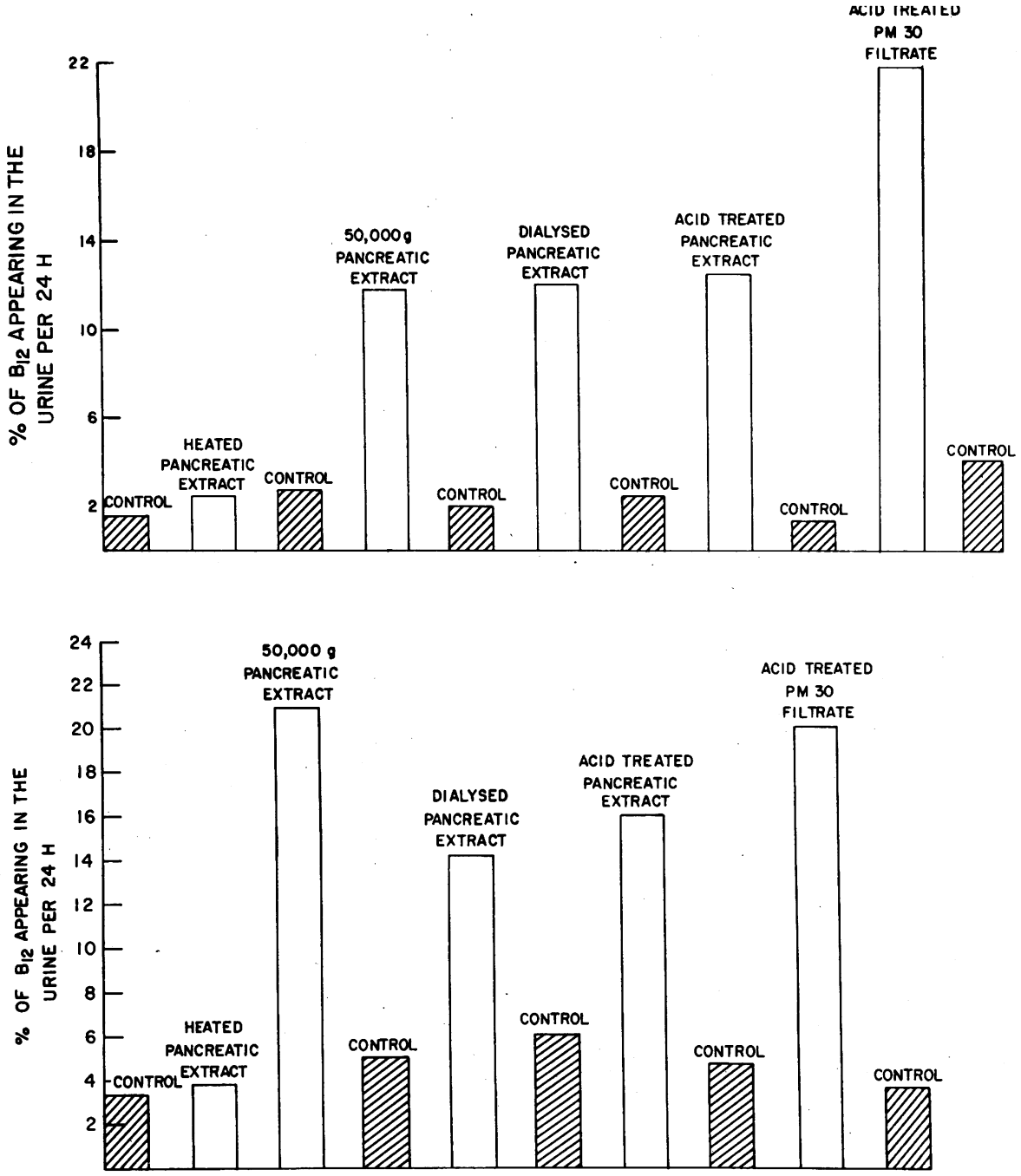

FIGURE 1 The urinary excretion of orally administered labeled vitamin B_{12} in two patients with pancreatic insufficiency. The effect of temperature, centrifugation, dialysis, acidification, and ultrafiltration on the vitamin B_{12}-promoting constituent of hog pancreatic extract.

in 20 ml of normal saline. The whole homogenate, containing 4,200 mg of protein, was centrifuged at 20,000 rpm (50,000 *g*) at 10°C for 30 min. The precipitate was discarded and the supernate, containing 1,800 mg of protein, was designated as the soluble fraction. The soluble fraction was then dialyzed against normal saline for 36 h at 4°C through a cellophane membrane,[7] and the retentate contained 299 mg of protein. In other experiments, the pH of the soluble fraction was brought to 1.5 for 30 min with 1 N HCl, the precipitate so formed was discarded, and

[7] Union Carbide Corp., Chicago, Ill.

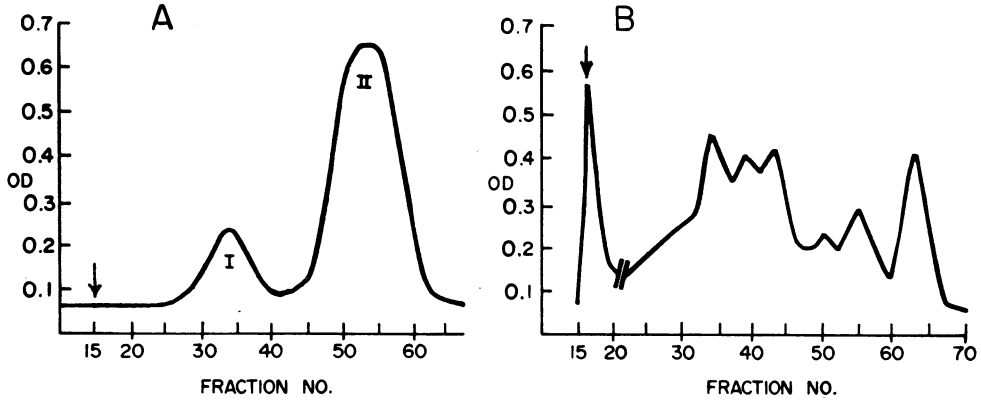

FIGURE 2 (*A*) Chromatography of hog pancreatic extract on Sephadex gel 100 media. The void volume is indicated by the vertical arrow. (*B*) Chromatography of hog pancreatic extract on Sephadex gel 25 media. The void volume is indicated by the vertical arrow.

the supernate brought to pH 7.0 with 1 N NaOH. The resultant soluble acid-stable fraction, containing 1,014 mg of protein, was passed through an Amicon PM-30 membrane[8] that allows for passage of spherical compounds of less than 30,000 in molecular weight. The filtrate obtained contained 105 mg of protein. In some experiments the crude extract was heated for 10 min at 100°C. Protein determinations were performed by the method of Lowry, Rosebrough, Farr, and Randall (7).

Chromatography of pancreatic extract. The soluble acid-stable fraction of pancreatic extract was applied to a 2.5 × 45 cm column packed with either Sephadex[9] Gel 100, G 50, or G 25 media and eluted with normal saline. In some experiments known substances were applied to the column as markers: ovalbumin,[9] blue dextran 2000,[9] chymotrypsin,[9] ribonuclease A,[9] and vitamin B_{12}.[10]

Enzymatic analysis of the soluble, acid-treated extract. Amylase was measured by the method of Somogyi (8), and lipase by the method of Cherry and Crandall (9). Trypsin was determined spectrophotometrically according to the procedure of Hummel (10) with *p*-toluenesulphonyl-L-arginine methyl ester (TAME)[11] as a substrate. Chymotrypsin was determined by the method of Schwert and Takenaka (11) with *N*-acetyl-L-tyrosine ethyl ester (ATEE)[12] as the substrate.

RESULTS

Effect of temperature, centrifugation, dialysis, acidification, and ultrafiltration on the vitamin B_{12}-promoting constituent of hog pancreatic extract. Two patients with pancreatic exocrine insufficiency and vitamin B_{12} malabsorption responsive to pancreatic extract served as subjects for assessing the vitamin B_{12}-promoting capacity of various subfractions of hog pancreas. As shown in Fig. 1, the constituent in the pancreatic extract that corrected vitamin B_{12} malabsorption was heat labile, present in the 50,000 *g* supernate, found in the retentate following dialysis across cellophane membranes, acid stable, and filterable through a membrane that retains spherical compounds greater than 30,000 in molecular weight.

Gel chromatographic fractionation of hog pancreatic extract. Fig. 2*A* shows the chromatographic behavior of the 50,000 *g* acid-treated extract on Sephadex G 100 media. As shown, two major peaks were obtained: a larger molecular weight fraction (peak I) and a smaller-sized fraction (peak II). Each peak was then applied separately to a Sephadex G 25 column and the results obtained are shown in Fig. 2*B*. Peak I appeared in the void volume and hence was composed of compounds of greater than 5,000 mol wt. Peak II yielded several peaks within the fractionation range of the G 25 column and thus contains compounds of less than 5,000 mol wt. In order to further characterize peak I, a portion was applied to a G 50 Sephadex column together with standards of known molecular weight. Peak I eluted at a fractionation volume in the range between chymotrypsin (mol wt 24,500) and ribonuclease (13,500).

The effect of chromatographic fractions of hog pancreatic extract on vitamin B_{12} absorption in partially pancreatectomized rats. Peaks I and II were administered concomitantly with labeled vitamin B_{12} to partially pancreatectomized rats with vitamin B_{12} malabsorption. As shown in Fig. 3, when peak I (containing 0.4 mg of protein) was administered to nine partially pancreatectomized rats whose vitamin B_{12} absorption was 73% of a simultaneously studied control group, absorption improved to a level similar to that observed in unoperated rats. When the partially pancreatectomized rats were restudied 2 wk later, vitamin B_{12} malabsorption was noted again. Peak II failed to improve vitamin B_{12} absorption.

Measurement of enzyme activities in hog pancreatic subfractions. As shown in Table I, heating the whole

[8] Scientific Systems Division, Amicon Corporation, Lexington, Mass.
[9] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[10] Smith, Miller, and Patch, New York.
[11] Calbiochem, Los Angeles, Calif.
[12] Sigma Chem Corporation, St. Louis, Mo.

TABLE I

Enzyme Activities of Subfractions of Hog Pancreas

Subfraction	Enzyme activity				Quantity protein administered	
	Trypsin	Chymotrypsin	Amylase	Lipase	Human subjects	Rats
	U/mg protein				*mg*	
Whole	147	352	16.8	0.78	4,200	—
Heat treated	0	0	0	0	3,800	—
50,000 *g** supernatant	260	460	6.0	0.96	1,800	—
Retentate after dialysis	439	800	93.0	13.8	299	—
Acid-treated	292	308	0	0	1,014	—
PM-30 filtrate	169	183	0	0	105	—
Peak I	3,950	2,800	0	0	—	0.4

* In some experiments the 50,000 *g* supernatant was dialyzed against normal saline through cellophane membranes while in other experiments the 50,000 *g* fraction was acid treated. This acid-treated subfraction was then carried through the remaining subfractionation procedures of ultrafiltration and chromatography.

extract destroyed all measured enzyme activity. Amylase and lipase activities were destroyed by lowering the pH to 1.5 for 30 min but trypsin and chymotrypsin activities remained relatively intact. Trypsin and chymotrypsin activities were present in the PM-30 and peak I fractions.

Effect of crystalline bovine trypsin on vitamin B_{12} absorption in partially pancreatectomized rats and patients with pancreatic insufficiency. 2 mg of bovine crystalline trypsin (180 TAME U/mg) were administered to 17 partially pancreatectomized rats which absorbed 50% as much vitamin B_{12} as a simultaneously studied control group of rats. Trypsin restored vitamin B_{12} absorption to levels observed in control rats. Fig. 4 demonstrates that 10 mg of crystalline trypsin (1,800 TAME U) corrected the vitamin B_{12} absorptive defect in one patient with pancreatic insufficiency and one patient with both pancreatic insufficiency and pernicious anemia.

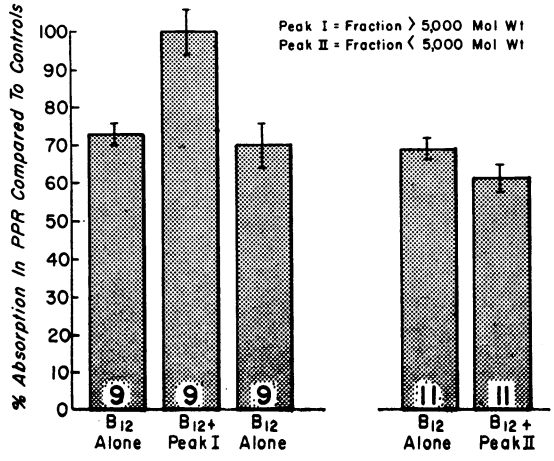

FIGURE 3 The effect of peaks I and II obtained from chromatography of hog pancreatic extract on vitamin B_{12} absorption in partially pancreatectomized rats. Absorption is expressed as the percent absorption in partially pancreatectomized rats compared to a group of simultaneously studied control rats. The results are expressed as the mean ±1 SE. Absorption in control rats ranged from 52.2±2.2% to 63.8±2.8%. Absorption was significantly improved with the administration of peak I ($P < 0.01$) when compared to the absorption of vitamin B_{12} alone.

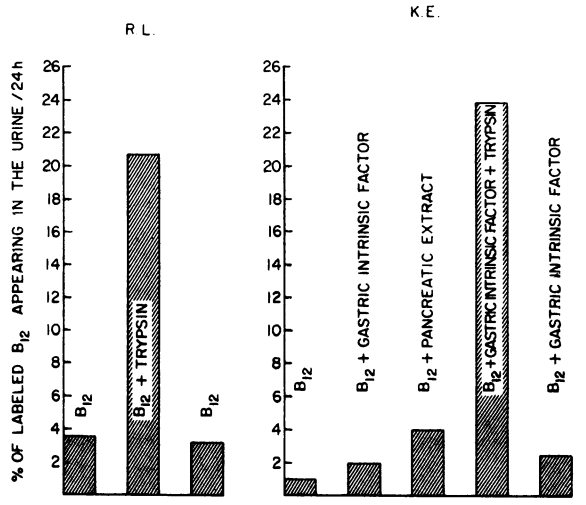

FIGURE 4 The effect of trypsin on the urinary excretion of orally administered labeled vitamin B_{12}. Patient R. L. has pancreatic insufficiency. Patient K. E. has both pernicious anemia and pancreatic insufficiency, requiring the administration of both gastric intrinsic factor and trypsin to correct the vitamin B_{12} malabsorption.

DISCUSSION

Pancreatic extract has been shown to correct the vitamin B_{12} malabsorption observed in some patients with pancreatic insufficiency (1, 2) and in rats with partial pancreatic extirpation (4). In previous studies the pancreatic supplement has been shown not to be contaminated with significant quantities of gastric intrinsic factor (2).

The data in this report demonstrates that the vitamin B_{12}-promoting constituent in pancreatic extract is soluble at 50,000 *g*, heat labile, acid stable, and approximately 20,000–25,000 in molecular weight. The pancreatic proteolytic (but not amylolytic or lipolytic) enzymes possess similar properties. In fact, crystalline trypsin administered to partially pancreatectomized rats and pancreatic insufficient subjects with vitamin B_{12} malabsorption improved the absorption of this vitamin. Whether the other proteolytic enzymes contained in the active subfractions (chymotrypsin and cathepsin) also possess the capacity to promote vitamin B_{12} absorption has not been evaluated. It is of interest to note that the quantity of crystalline trypsin administered (10 mg) is of the same magnitude that can be secreted by the human pancreas within several minutes following secretin or cholecystokinin administration (12), and about 5% of the daily trypsin output in the ileal effluent of patients with ileostomies (13).

The improvement in vitamin B_{12} absorption following sodium bicarbonate administration reported previously (1) had been interpreted to indicate that bicarbonate raises the intraluminal pH of the ileum and thus creates a more favorable environment for attachment of the gastric intrinsic factor-vitamin B_{12} complex onto the ileal mucosa. However, our previous studies have indicated that the ileal pH of patients with pancreatic insufficiency and vitamin B_{12} malabsorption is no different from the ileal pH found in control subjects (2). It is of interest to note that the pH optimum for tryptic activity is 7–8 (14) and hence sodium bicarbonate may provide a more conducive environment to allow for adequate activity of the residual trypsin that is still secreted in humans or rats with pancreatic insufficiency.

The mechanism by which trypsin improves vitamin B_{12} absorption remains to be defined. Gastric juice from patients with pancreatic insufficiency contains immunoreactive intrinsic factor (2) and exogenous gastric intrinsic factor does not correct vitamin B_{12} malabsorption in patients with pancreatic insufficiency (1, 2). Gastric homogenates from partially pancreatectomized rats can stimulate vitamin B_{12} uptake in rat intestinal sacs (4). In addition the small intestine obtained from partially pancreatectomized rats maintains its ability to respond to gastric intrinsic factor (4). Thus it appears that neither gastric intrinsic factor nor the ileal receptor requires the presence of a pancreatic factor to be biologically active. Rather, the pancreatic factor may function within the lumen of the gastrointestinal tract to maintain the gastric intrinsic factor-vitamin B_{12} complex in a form readily available for absorption.

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