Trypsin-Like Nature of the Pancreatic Factor
That Corrects Vitamin B₁₂ Malabsorption
Associated with Pancreatic Dysfunction

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ABSTRACT Hog pancreas was subfractionated and
assessed for its ability to correct vitamin B₁₂ malabsorption
in patients with pancreatic dysfunction and in rats
with partial pancreatic extirpation. The constituent
obtained from the pancreas that increased vitamin B₁₂
absorption in both humans and rats was soluble at 50,000
g, heat labile, acid stable, and approximately 20,000-
25,000 in molecular weight. The active subfractions
contained trypsic and chymotryptic but no amylas eor
lipase activity. Trice-crystallized trypsin corrected the
vitamin B₁₂ malabsorption in both patients with pancreatic
insufficiency and in rats with subtotal pancreatec-
tomy. These data indicate that pancreatic proteolytic en-
yzmes—in particular, trypsin—are necessary for optimal
vitamin B₁₂ absorption.

INTRODUCTION
Vitamin B₁₂ malabsorption occurs in some patients with
chronic pancreatic exocrine insufficiency (1-3) and in
rats subjected to partial pancreatectomy (4). Although
hag pancreatic extract corrects the absorptive defect in
both humans and animals (1, 2, 4), the precise constitu-
ent in the pancreatic extract responsible for the enhance-
ment of vitamin B₁₂ absorption has not been determined.

The present study (a) evaluates the ability of sub-
frations of hog pancreatic extract to correct vitamin B₁₂
malabsorption in patients with pancreatic insufficiency
and in partially pancreatectomized rats, (b) correlates the
capacity of pancreatic subfractions to correct vitamin
B₁₂ malabsorption with the concentrations of various pan-
creatic enzymes in the subfractions, and (c) demon-
strates that crystalline trypsin possesses the capacity to
enhance the vitamin B₁₂ malabsorption associated with
pancreatic dysfunction.

METHODS
Vitamin B₁₂ absorption studies in human subjects. Ab-
 absorption was measured by the standard urinary excretion
test using 0.5 μg of ⁶⁷Co-labeled vitamin B₁₂ (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 subfrac-
tions or trypsin¹ were administered concomitantly with
labeled vitamin B₁₂.

Vitamin B₁₂ absorption studies in rats. Partial pan cre-
tectomies 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 ⁶⁷Co-labeled vita
min B₁₂ containing 5 ng of vitamin B₁₂ (13-15 μCi/μg)
was administered via gastric tube to fasted rats. Immedi-
ately after dosing and again 6 days later, whole body radio-
activity 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 radio-
activity on day 1 times 100. Pancreatic subfractions and
trypsin were administered with labeled vitamin B₁₂ to cer-
tain animals.

Preparation of pancreatic subfrations. 9.6 g of hog pan-
creatic extract (Viokase⁵ or Cotzyme⁶) were homogenized

¹ Worthington Biochem Corp., Freehold, N. J.
² Charles River CD Rats, Charles River Breeding Labo-
 ries, 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 ViBin Corp., Monticello, Ill.
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, 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

*Union Carbide Corp., Chicago, Ill.*
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* 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 x 45 cm column packed with either Sephadex* G 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, blue dextran 2000, chymotrypsin, ribonuclease A, and vitamin B_{12}.

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) as a substrate. Chymotrypsin was determined by the method of Schwert and Takenaka (11) with N-acetyl-L-tyrosine ethyl ester (AT-EE) 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. 2A 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. 2B. 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

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* Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
* Smith, Miller, and Patch, New York.
* Calbiochem, Los Angeles, Calif.
* Sigma Chem Corporation, St. Louis, Mo.
TABLE I

Enzyme Activities of Subfractions of Hog Pancreas

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>Enzyme activity</th>
<th>Quantity protein administered</th>
</tr>
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<tbody>
<tr>
<td>Whole</td>
<td>Trypsin 147</td>
<td>U/mg protein</td>
</tr>
<tr>
<td></td>
<td>Chymotrypsin 352</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amylase 16.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipase 0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human subjects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rats</td>
</tr>
<tr>
<td>Heat treated</td>
<td>0</td>
<td>4,200</td>
</tr>
<tr>
<td>50,000 g* supernatant</td>
<td>260</td>
<td>3,800</td>
</tr>
<tr>
<td>Retentate after dialysis</td>
<td>439</td>
<td>1,800</td>
</tr>
<tr>
<td>Acid-treated</td>
<td>292</td>
<td>299</td>
</tr>
<tr>
<td>PM-30 filtrate</td>
<td>169</td>
<td>1,014</td>
</tr>
<tr>
<td>Peak I</td>
<td>3,950</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

* 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.

The effect of crystalline bovine trypsin on vitamin B₁₂ 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 B₁₂ as a simultaneously studied control group of rats. Trypsin restored vitamin B₁₂ absorption to levels observed in control rats. Fig. 4 demonstrates that 10 mg of crystalline trypsin (1,800 TAME U) corrected the vitamin B₁₂ 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 B₁₂ 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 B₁₂ alone.

FIGURE 4 The effect of trypsin on the urinary excretion of orally administered labeled vitamin B₁₂. 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 B₁₂ malabsorption.
DISCUSSION
Pancreatic extract has been shown to correct the vitamin B₉ malabsorption observed in some patients with pancreatic insufficiency (1, 2) and in rats with partial pancreatectomy (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 B₉-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 B₉ 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 B₉ 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 B₉ 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 B₉ complex onto the ileal mucosa. However, our previous studies have indicated that the ileal pH of patients with pancreatic insufficiency and vitamin B₉ malabsorption is no different from the ileal pH found in control subjects (2). It is of interest to note that the pH optimum for trypsin 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 B₉ 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 B₉ malabsorption in patients with pancreatic insufficiency (1, 2). Gastric homogenates from partially pancreatectomized rats can stimulate vitamin B₉ 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 B₉ complex in a form readily available for absorption.

REFERENCES