Physiol., 1944, v144, 326.

12. Dworkin, R. M., Proc. Soc. Exp. Biol. and Med., 1944, v56, 20.

13. Handford, S. W., Smythe, C. M., Gilmore, J. P., Leopold, R. S., Kathan, R. H., Arm, H. G., J. Appl. Physiol., 1955, v7, 553.

14. Miyagawa, Y., Japan. J. Exp. Med., 1933, v23, 59.

15. Wiggers, C. J., Physiology of Shock, New York, Commonwealth Fund, 1950.

16. Schecter, I., Hestrin, S., J. Lab. Clin. Med., 1963, v61, 962.

17. Symposium on Intravenous Fat Emulsions, Metabolism, 1957, v6, 591.

18. Di Palma, J. R., Ed., Drill's Pharmacology in Medicine, 3rd ed., McGraw-Hill, New York, 1965.

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Enhanced Vitamin B_{12} Absorption from Rat Intestine by Proteases In the Absence of Intrinsic Factor. (31671)

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In our search for a readily absorbable form of vitamin B_{12} complex produced during the digestive process of liver, it was found that B_{12} in liver was absorbed from an isolated rat intestine to an extent comparable to that achieved with free B_{12} and homologous intrinsic factor (IF) when Pronase(1), a Streptomyces proteases preparation, was used for digestion. The effect of Pronase which was independent of IF, was demonstrable whether digestion was carried out in test tubes or inside the intestine, as long as it was present in the intestine at the time of absorption. No other pure proteases tested had comparable effects. Absorption of crystalline B_{12} of small doses was similarly enhanced by Pronase from an isolated rat intestine in the absence of IF. Pronase, a mixture of several proteolytic enzymes and one of the most potent preparations available, was used for the purpose of simulating, *in vitro,* the intestinal digestion which is much more efficient than any artificial digestion system using a single crystalline enzyme.

So far, no other compounds have been shown to enhance absorption of small doses of B_{12} under a condition where IF is lacking, and the present observations seem to provide important clues as to the mechanism of intestinal absorption of B_{12} in relation to IF action. This is a preliminary report of our finding.

Methods. Co^{57}-hydroxocobalamin* (Co^{57}-B_{12}) with a specific activity of approximately 8 $\mu c/\mu g$ was injected subcutaneously to adult rats of the Wistar strain, 10 $m\mu g$ daily, for 3 weeks and the liver was removed 4 days after the last injection. The liver was cut into small pieces, dried *in vacuo* over P_2O_5 at 4°C and pulverized. The isotopic dilution of Co^{57}-B_{12} in the material was 50- to 90-fold as determined by the microbiological assay using the Skeggs' medium(2) and *Lactobacillus leichmanii* 4797, and by the radiometric measurement with a gamma scintillation counter. In some experiments, instead of dried liver, fresh liver homogenate was used. It was assumed that most of the radioactivity in such liver preparations represented B_{12} in its natural form(3).

Enzymatic digestion of liver was carried out *in vitro* using crystalline trypsin or a mixture of equal portions of trypsin, α-chymotrypsin and papain in pH 7.6 tris-maleate buffer, Pronase-P† in pH 7.4 phosphate buffer, or pepsin in 1/20 N HCl, in a water bath at 37°C under agitation, as well as *in vivo* using Pronase and trypsin. After digestion *in vitro,* the mixture was centrifuged and the supernatant was applied quantitatively in a rat intestinal loop for absorption measurement.

The effect of IF and digestion on absorption was studied by the intestinal loop meth-

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† Purchased from Kaken Chemical Co., Ltd., Bunkyo-Ku, Tokoyo. Activity: 45,000 P.U.K./g.

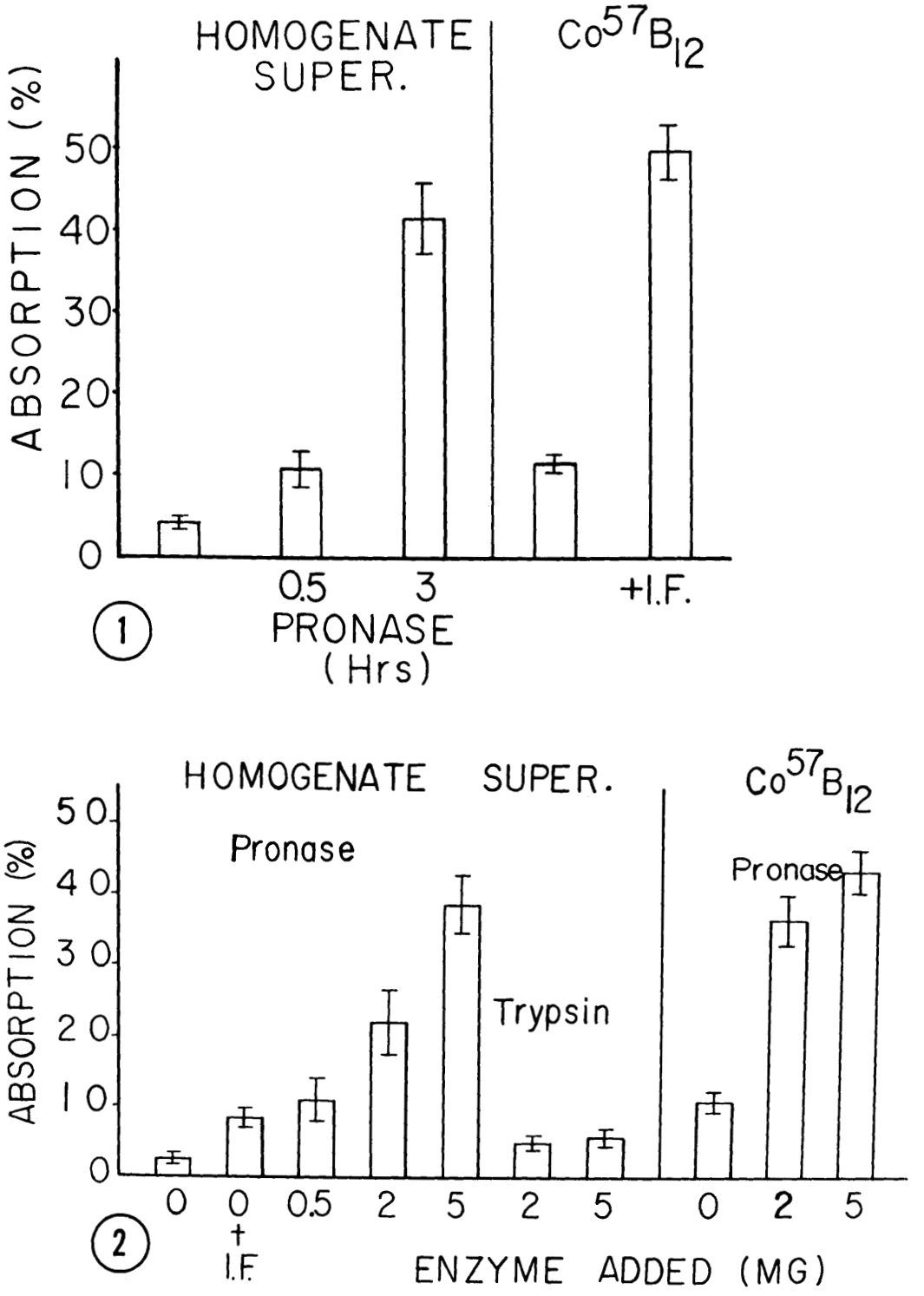

FIG. 1. Absorption after Pronase digestion, of Co^{57}-B_{12} in liver homogenate in comparison with free Co^{57}-B_{12}. Each dose contained 20 mμg Co^{57}-B^{12} or its equivalent; 1 mg of Pronase was

mixed with 1.5 ml of 10% fresh liver homogenate. Vertical bars represent standard deviations. Each group consisted of 4 loops.

FIG. 2. Absorption of Co^{57}-B_{12} in liver homogenate supernatant or free Co^{57}-B^{12} when applied in the rat intestinal loop together with various amounts of Pronase or trypsin. Each group consisted of 4 loops, and received a dose containing 20 mμg Co^{57}-B_{12} or its equivalent in homogenate supernatant. Vertical bars represent standard deviations.

od(4). This technique involves the preparation of an isolated loop of the small intestine below the duodenojejunal junction, which has been washed free of IF with saline and receives no endogenous IF. Absorption of radioactive B_{12} applied with or without IF is measured from the decrease of radioactivity from the loop after 16-24 hours. Under these conditions a linear correlation is demonstrable between the dose of IF and enhancement of absorption. As the source of IF, a saline extract of fresh rat gastric mucosa(4) was used in amounts equivalent to 2-5 times the B_{12} dose in terms of binding powder determined by the dialysis method(5).

Results. Liver powder was suspended in a buffer or acid, 50 mg per ml, and one of the enzymes or their mixture was added at the ratio of 1:100 by weight as described, hydrolyzed at 37°C for 0.5, 3 and 5 hours, and the supernatant, neutralized in the case of pepsin, was applied into the loop in a dose equivalent of 10 to 30 mμg of B_{12}. After 3 hours of hydrolysis, more than 80% of the radioactivity in the original powder was released into the supernatant and most of the radioactivity in the supernatant was found to be dialyzable. Pronase proved to be most potent in the digesting capacity, but the difference among these enzymes became small after 3 hours of incubation. When tested in the loop, absorption of radioactivity in the supernatant was not superior to that of crystalline Co^{57}-B_{12}, and coadministration of IF enhanced absorption of both crystalline Co^{57}-B_{12} and Co^{57}-B_{12} in digested liver (Table I).

We then used a fresh 10% homogenate of radioactive liver and absorption of radioactivity in the supernatant after digestion was similarly measured. When the supernatant from 1.5 ml of homogenate after 3 hours digestion with 1 mg of Pronase, was applied to the loop, absorption was significantly increased (Fig. 1). It was suspected that an absorbable form of B_{12}-peptide complex was produced during digestion with Pronase. In order to subject such digestion products to absorption as they were liberated, supernatant of 1.5 ml of the homogenate was mixed with graded doses of Pronase or trypsin and applied into loops without test tube digestion. As in Fig. 2, absorption of radioactivity in the supernatant was definitely increased when 2

TABLE I. Absorption of Liver Bound and Crystalline Co^{57}-B_{12} from Rat Intestinal Loops.

Material	Digestion	No. of loops	Radioactivity recovered (%) Loop	(Absorption)	Liver + kidneys
Liver powder†	Trypsin, 0.5 hr	3	92.4 ± 3.1*	(7.6)	3.8
	" , 3 "	3	95 5 ± 2.1	(4.5)	2.1
	" , 5 "	3	96.1 ± 2.9	(3.9)	1.9
	Pepsin, 3 "	3	93.6 ± 2.5	(6.4)	3.2
	" , 5 "	3	96.6 ± 3.2	(3.3)	1.8
	Trypsin, chymotrypsin & papain, 3 hr	3	98.5 ± 2.9	(1.5)	1.0
	Pronase, 3 hr	3	91.5 ± 3.1	(8.5)	4.1
Liver powder + IF‡ (added after digestion)	Trypsin, 3 hr	3	53.6 ± 2.7	(46.4)	21.1
Co^{57}-B_{12}	—	4	89.0 ± 1.5	(11.0)	5.6
Co^{57}-B_{12} + IF‡	—	4	51.8 ± 3.2	(48.2)	20.3

* Avg ± standard error.
† Supernatant of 50 mg of powder, equivalent of 10-30 mμg B_{12} per dose.
‡ Capable of binding 50 mμg B_{12}.

TABLE II. Absorption of Co^{57}-B_{12} in Digested Liver, After Heat Inactivation of Pronase or Dialysis.

Material	Treatment	Absorption from loop (%)	Uptake by liver and kidneys (%)
Liver powder, digested with 5 mg Pronase*	Heating†	4.6	2.8
	—	39.2	20.2
	Dialysis‡	5.4	2.9
	" + IF	50.4	24.1
Liver homogenate, digested with 5 mg Pronase*	Heating	7.2	3.9
	—	41.3	23.2

Figures are averages of 2 to 3 loops. Each dose was equivalent of about 15 mμg B_{12}.
* Digested for 3 hr.
† 20 min at 100°C.
‡ Ultrafiltrable portion was lyophilized and used.

TABLE III. Sequential Application of Pronase and Co^{57}-B_{12} to the Loop and Effect of Removal of the Former on Absorption of the Latter.

Time relation of application and loop washing	Absorption from loop (%)	Uptake by liver and kidneys (%)
5 mg Pronase—30 min—Washing, Co^{57}-B_{12}*	5.8	3.5
" — 1 hr—Washing, Co^{57}-B_{12}	4.9	2.6
" — 1 hr—Washing, Co^{57}-B_{12} + IF†	53.2	22.4
" — 1 hr—Co^{57}-B_{12}	49.2	20.9

Figures are averages of 2 to 3 loops. * 10 mμg. † Capable of binding 30 mμg B_{12}.

mg Pronase was used, and the increase was more marked with 5 mg and less apparent with 0.5 mg of Pronase, while the same doses of trypsin failed to so the same. Very similar results were obtained with supernatant of liver powder suspension and Pronase. Subsequently, it became clear that the Pronase effect was also demonstrable with free Co^{57}-B_{12} as shown in the right compartment of the same Figure, and that as long as more than 2 mg of Pronase was placed in the loop, no previous digestion was necessary.

To inactivate Pronase, the digestion mixture of either liver powder or homogenate and 5 mg Pronase was heated at 100°C for 20 minutes after 3 hours of incubation. When the supernatant was tested in the loop, no enhanced absorption occurred (Table II). In another experiment, Pronase digestion was carried out with liver powder under the same condition except that it was done in a cellulose bag immersed in distilled water in a test tube, to separate ultrafiltrable digestion products. The dialyzed portion was lyophilized and applied to the loop. No enhancement of absorption was demonstrated as shown in the same Table. These results clearly indicated that the absorption enhancement was attributable to Pronase, not to an unknown, readily absorbed, digestion product.

We then wished to see if Pronase altered the mucosa in such a way that free B_{12} easily crossed the barrier. To this end, 5 mg of Pronase in 1 ml of water was placed in the loop, the abdomen closed, the loop was washed again with saline after 30 minutes or 1 hour to remove Pronase, and then Co^{57}-B_{12} with or without IF was applied. No effect of Pronase was demonstrable any more and IF enhanced absorption as usual. Without rewashing, Pronase exerted the same effect on the absorption of Co^{57}-B_{12} applied after one hour interval (Table III).

Discussion. These results were obtained from *in vivo* studies using isolated rat intestine where IF had been removed. Application of 2-5 mg of Pronase in such intestine markedly increased absorption of physiological amounts of crystalline Co^{57}-B_{12} or Co^{57}-B_{12} liberated from liver by digestion, and the increase was almost the same as that achieved by coadministration of homologous IF. The bulk of radioactivity in liver after repeated injections of Co^{57}-B_{12} would represent the natural form of this vitamin, probably cobalamin coenzyme(3,6,7), and

that in the supernatant after digestion, hydroxocobalamin(7). Interpretation of the results does not depend on the chemical form of B_{12}, since there is no significant difference in its requirement of IF and rate of absorption between cyano-, hydroxo- and coenzyme form of B_{12}(7,8,9).

The isolated loop used in this study did not have the influx of pancreatic juice and therefore lacked the natural digestive capacity. Pronase was used primarily for supplementing the digestive power in the loop to simulate natural intestinal digestion. It may be of interest to note that pernicious anemia and post-total gastrectomy state have been the main target for the correction of defective B_{12} absorption, and the gastrointestinal digestion is markedly impaired in both conditions. Heathcote and Mooney have held that the reduced gastric proteolysis is the major defect in pernicious anemia that is responsible for B_{12} deficiency(10,11). Furthermore, it has been shown repeatedly that in some cases of pancreatic insufficiency defective B_{12} absorption is not corrected by addition of IF (12,13,14); it was corrected by administration of bicarbonate which improves pancreatic digestion by raising duodenal pH(13).

D-sorbitol has been shown to enhance absorption of B_{12} given in large doses in the presence of IF(15), but it does not increase absorption of physiological amounts of B_{12} (16) although it forms a complex with B_{12} *in vitro*(17). The mode of action by which Pronase enhances absorption of B_{12} is not clear. It might prevent formation of an unabsorbable form of B_{12} in the gut, inactivate inhibitors, alter the mucosal permeability, etc. Whatever the action, it is of significance that absorption of B_{12} could be modified by digestive processes inside the gut. There may be a similar but hitherto unexplored phase in the physiological absorption of B_{12}, and the previous studies on the action of IF may have to be reevaluated in the light of the present finding.

Summary. Rat liver containing radioactive B_{12} in its natural form was prepared by repeated injections of Co^{57}-hydroxocobalamin and absorption of liver bound B_{12} was investigated using isolated rat intestine *in vivo*. There was no indication for the production of readily absorbable forms of B_{12} complex during digestion with trypsin, chymotrypsin, papain, pepsin and Pronase. It was found that Pronase, a potent Streptomyces proteases preparation, increased absorption of liver bound B_{12} as well as free B_{12} in the absence of IF, the increase being comparable to that obtained with IF under the same condition. This effect was demonstrable only when Pronase was placed in the isolated intestine in large quantities. These results suggest a possible relationship of intestinal digestion to the absorption mechanism of B_{12} which involves IF.

1. Nomoto, M., Narahashi, Y., J. Biochem., 1959, v46, 653.

2. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., Wright, L. D., J. Biol. Chem., 1950, v184, 211.

3. Toohey, J. I., Barker, H. A., ibid., 1961, v236, 560.

4. Okuda, K., Am. J. Physiol., 1960, v199, 84.

5. ———, Clin. Chim. Acta, 1962, v7, 780.

6. Rosenblum, C., Williagan, D. A., Meriwether, H. T., Cronkite, E. P., Proc. Soc. Exp. Biol. and Med., 1960, v105, 142.

7. Rosenblum, C., Vitamin B_{12} und Intrinsic Factor, Enke, Stuttgart, Germany, 1962, 306.

8. Okuda, K., Tantengco, V., Proc. Soc. Exp. Biol. and Med., 1962, v110, 396.

9. Chosy, J. J., Killander, A., Schilling, R. F., Vitamin B_{12} und Intrinsic Factor, Enke, Stuttgart, Germany, 1962, 668.

10. Heathcote, J. G., Mooney, F. S., Lancet, 1958, v1, 982.

11. ———, Brit. Med. J., 1961, v1, 232.

12. McIntyre, P. A., Sachs, M. V., Krevans, J. R., Conley, C. L., Arch. Int. Med., 1956, v96, 541.

13. Nieweg, H. O., Abels, J., Veeger, W., Hellemanns, N., Vitamin B_{12} und Intrinsic Factor, Enke, Stuttgart, Germany, 1962, 157.

14. Perman, G., Gullberg, R., Reizenstein, P. G., Snellman, B., Allgen, L. G., Acta Med. Scand., 1960, v168, 117.

15. Chow, B. F., Meier, P., Free, S. M., Jr., Am. J. Clin. Nutr., 1958, v6, 30.

16. Okuda, K., Proc. Soc. Exp. Biol. and Med., 1961, v108, 816.

17. Heinrich, H. C., Klin. Wochschr., 1959, v37, 525.

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