increased absorption, however, when subjected to reduced atmospheric pressure or oxygen tension, a situation very similar to that seen with hypophysectomized animals.

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Releasing Factor and Endogenous Vitamin B_{12}* (30240)

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The existence of a "releasing factor" capable of splitting vitamin B_{12} from its complex with intrinsic factor has been considered since the report by Ungley(1) who stated that a substance contained in the human small intestine could remove vit B_{12} from its complex with normal human gastric juice.

Cooper and Castle(2) found that rat intestinal extract contained "releasing factor" activity which split the bond between vit B_{12} and rat intrinsic factor and Doscherholmen(3) confirmed their findings. Herbert, Cooper, and Castle(4) questioned the physiologic importance of this vit B_{12} releasing factor when they were able to demonstrate releasing factor activity only in the proximal area of the rat's small intensine and not in the mid-ileum where vit B_{12} absorption takes place(5). It was later shown by Ellenbogen and Highley(6) that releasing factor activity was present in the mid-ileum as well as in the proximal area of the rat's small intestine.

A releasing factor was noted in various human organ extracts by Nyberg, Saarni, and Gräsbeck(7). Perlman, Guiffre, and Amrein (8) reported the displacement of radioactive vit B_{12} bound by mouse ascitic fluid or hog intrinsic factor by unbound non-radioactive vit B_{12}.

The experiments reported here were performed for the purpose of grossly identifying the substance(s) responsible for the release of vit B_{12} by rat intestinal extracts and extracts of various human organs. In a complex equilibrium system, endogenous vit B_{12} contained in rat intestinal extracts exchanges with intrinsic factor-bound radioactive vit B_{12}, as suggested by Donaldson and Katz (9). This mechanism appears to account for all the releasing activity exhibited by the mid-ileum and for a major portion, and perhaps all, contained in the proximal area, as well as the releasing effect shown by human organ extracts.

Materials and methods. Vitamin B_{12} labeled

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TABLE I. Effect of Dialysis of Releasing Factor Extracts.

Portion of small intestine	Vit B_{12} released* Before dialysis	Vit B_{12} released* After dialysis	Vit B_{12} content Before dialysis	Vit B_{12} content After dialysis
	(%)	(%)	(mμg)	(mμg)
1. Proximal end	29.8	13.5	14.6	9.4
2.	0	1.6	7.9	6.2
3.	7.3	2.9	8.9	7.5
4.	15.5	8.5	13.0	6.2
5.	18.5	-3.3	13.3	10.1
6.	17.8	1.6	10.4	8.6
7.	19.5	7.7	8.6	5.8
8.	15.1	7.8	9.5	4.9
9.	13.9	0	5.7	4.6
10. Ileal end	0	0	4.6	4.8

* Corrected for control (vit B_{12}-rat IF without intestinal extract).

with CO^{60} and CO^{57} was in part obtained from Merck and Co., Rahway, N. J., and in part from Dr. Elmer Alpert of Merck, Sharp and Dohme Research Laboratories. Measurement of radioactivity was performed in a sodium iodide well or plastic scintillator previously described(10). Carworth CFE and Sprague-Dawley rats were used in the preparation of stomach and intestinal extract(2,4, 6). For preparation of releasing factor from beef small intestine, the mucosa was scraped from twenty-six 15-inch segments of beef intestine taken at regular intervals from pylorus to caecum. The mucosa was homogenized in 0.07 M phosphate buffer at pH 7.6 or saline and used in place of rat intestinal homogenate in some experiments. The method of Nyberg, Saarni, and Gräsbeck(7) was used to prepare releasing factor of human tissues. The equilibrium techniques for measuring releasing factor activity have been reported(2,4,6). Endogenous vit B_{12} was determined by the U.S.P. vit B_{12} microbiological assay using *L. leichmanii,* A.T.C.C. no. 7380 and the vit B_{12} assay using *E. gracilis* as described by Ross(11).

Results. Intestinal segment extracts 1 to 10 obtained from Carworth CFE rats were dialyzed for 24 hours at 10°C against running tap water prior to assay. Three different preparations were compared for releasing factor activity before and after dialysis. Results obtained with one of these are shown in Table I. Although the 3 preparations gave comparable results, considerable variation was observed with individual segments, particularly after dialysis. The activity of the proximal area and mid-ileum was reduced after dialysis. Some negative values were obtained after dialysis of the segment extracts. This results when the quantity of radioactive vit B_{12} made dialyzable is less than the control sample (vit B_{12}-rat stomach extract to which no intestinal segment extract is added). This was presumably due to removal of bound vit B_{12} from the intestinal extracts during exhaustive dialysis. The binding sites from which the vit B_{12} was thus removed were capable of binding some of the vit B_{12} that was originally bound to the rat stomach extract. It can also be seen in Table I that the distribution of endogenous vit B_{12} in the rat small intestine was similar to that of releasing factor activity. However, agreement was poor between removal of endogenous vit B_{12} and loss of releasing factor activity during dialysis.

The possibility was suggested by the above data that endogenous vit B_{12} could have been responsible for the observed release of radioactive vit B_{12} by the intestinal extracts. It has been demonstrated with both human and rat gastric juice that substantial equilibrium exists at 37°C between bound and unbound vit B_{12}(9). Since the releasing factor assay is performed under equilibrium conditions for 6 hours at 37°C, it appeared possible that endogenous vit B_{12} of the intestinal extracts was equilibrating with radioactive vit B_{12} bound to the rat stomach extract. Nonradioactive cyanocobalamin was therefore substituted for the endogenous vit B_{12} contained in the intestinal segment extracts (Table II). Equilibration of radioactive vit B_{12} with the added unbound vit B_{12} did occur and, with the exception of segment 2, results were comparable to those obtained with the segments themselves.

Loss of releasing factor activity by exhaustive dialysis could have been caused by removal of endogenous vit B_{12} or by inactivation of a labile component. In the latter case, loss of endogenous vit B_{12} paralleling loss of releasing factor activity would have been coincidental. To study this, the releasing factor

TABLE II. Releasing Factor Activity of Endogenous Vitamin B_{12}.

Portion of small intestine	Vit. $B_{12}Co^{60}$ released with segments	Vit. $B_{12}Co^{60}$ released with cyanocobalamin
	(%)	(%)
1. Proximal end	37.3	31.8
2.	0	21.6
3.	9.6	24.0
4.	19.8	30.0
5.	22.5	27.8
6.	20.2	24.0
7.	20.6	18.5
8.	20.2	21.2
9.	10.6	13.9
10. Ileal end	5.8	8.7

TABLE III. Releasing Factor Activity of Intestinal Extracts Placed Outside Dialysis Membrane.

Portion of small intestine	Vit. $B_{12}Co^{60}$ released
	(%)
1. Proximal end	41.7
2.	11.9
3.	14.7
4.	24.3
5.	23.7
6.	30.2
7.	27.9
8.	20.5
9.	19.8
10. Ileal end	12.8

assay was modified by placing 2 ml of the intestinal segment extract on the outside of the dialysis membrane so that whatever was responsible for the release of vit B_{12} would have to pass through the membrane to exert its effect. It can be seen in Table III that activity comparable to that found in the usual method of assay was observed although it was somewhat higher. The latter finding may result from the slight vit B_{12} binding capacity of the segment extracts. When the extracts were placed within the dialysis membrane they bound some of the vit B_{12} originally bound to the rat stomach extract.

To study further the effect of endogenous vit B_{12}, the amount of the vitamin lost during exhaustive dialysis was added back to the dialyzed segment extracts. Activity lost in dialysis was restored with all the segments.

To determine if endogenous vit B_{12} was *solely* responsible for the observed release of radioactive vit B_{12}, increments of vit B_{12} (2.0 to 25 mμg) were used in place of the intestinal extracts in the releasing factor assay and a "standard curve" of the effect of unbound vit B_{12} was prepared (Fig. 1). Using this standard curve, the per cent of vit B_{12} released by the extracts was used to calculate their theoretical vit B_{12} content. If this value agreed with that obtained by microbiological assay, it would constitute presumptive evidence that vit B_{12} was solely responsible for releasing factor activity. In Table IV it is evident that agreement between observed and calculated values was excellent for segments 4, 5, and 6. However, the vit B_{12} content of segment 1 could account for only 60% of the activity displayed by the segment extract.

In Table V the releasing factor activity of

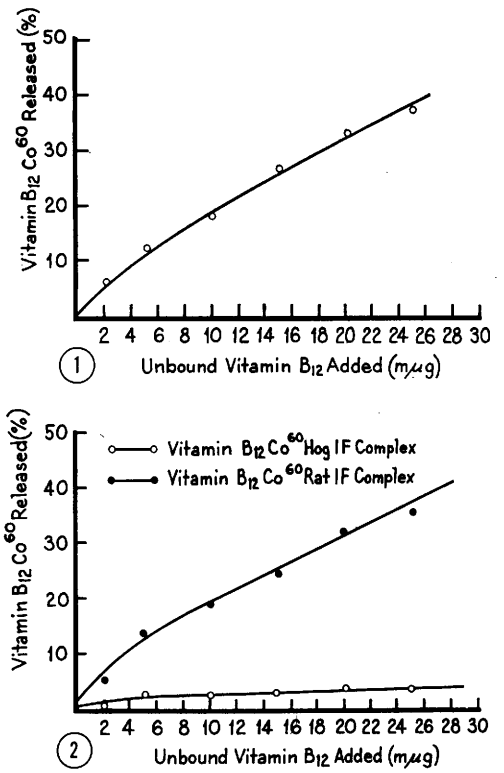

FIG. 1. A "standard curve" of vit. $B_{12}Co^{60}$ release from its rat intrinsic factor complex by unbound vit. B_{12}.

FIG. 2. Comparison of removal of vit. $B_{12}Co^{60}$ from rat or hog intrinsic factor (IF) complex by unbound vit. B_{12}. From 2 to 25 mμg of unbound cyanocobalamin equilibrated with 25 mμg of vit. $B_{12}Co^{60}$ bound to either rat or hog intrinsic factor. Temp. 37°. Time of incubation 6 hr.

TABLE IV. Quantitation of Releasing Factor Activity of Endogenous Vitamin B_{12}.

Portion of small intestine	Cyanocobalamin	Vit. $B_{12}Co^{60}$ released	Vit. B_{12} content of intestinal extract	
			Calculated	Found
	(mμg)	(%)	(mμg)	(mμg)
1. Proximal end	—	29.2	17.5	11.0
2.	—	20.5	10.2	7.6
3.	—	6.3	2.0	9.0
4.	—	20.2	10.0	11.0
5.	—	19.2	9.2	11.0
6.	—	22.3	11.6	12.0
7.	—	7.0	2.3	9.8
8.	—	7.0	2.3	9.8
9.	—	4.7	1.7	7.5
10. Ileal end	—	8.2	2.9	8.4
—	2.0	5.7	—	—
—	5.0	14.0	—	—
—	10.0	18.5	—	—
—	15.0	25.5	—	—
—	20.0	33.2	—	—
—	25.0	37.1	—	—

TABLE V. Releasing Factor Activity of Vitamin B_{12} Analogues.

Analogue tested	Vit. $B_{12}Co^{60}$ released		Clinical activity*
	(mμg)	(%)	(%)
1. Cyanocobalamin (10 mμg)	3.3	20.3	100
2. Pseudo B_{12} (10 mμg)	0	0	0
3. Factor III (10 mμg)	1.5	9.1	25–50
4. Factor A (10 mμg)	0	0	25
5. 5,6-dimethylbenzimidazolyl cobamide coenzyme (10 mμg)	1.9	11.8	100
6. Benzimidazolyl B_{12} (10 mμg)	3.0	18.3	100

* Using cyanocobalamin as standard.

analogues of vit B_{12} is compared with that of cyanocobalamin. The benzimidazole derivative was about as active as cyanocobalamin and 5,6-dimethylbenzimidazolyl cobamide coenzyme was about half as active. Aside from the coenzyme, agreement was good between releasing factor and clinical activities.

Mucosa homogenates from beef small intestine were substituted for rat intestinal homogenates in several experiments. These homogenates showed considerable releasing effect. The results are comparable to those found with rat intestinal homogenates.

Human organ tissue was obtained at autopsy within 30 minutes after cardiac arrest in an apparently healthy person. The tissue was frozen until extracts were prepared by the method of Nyberg *et al*(7). The releasing activity of these extracts showed correlation with the amount of endogenous vit B_{12} present. The amount released was no higher and often less than that predicted from the "standard curve" on the basis of native B_{12} found in the organ extracts (Table VI).

Rat and beef intestinal extracts release radioactive vit B_{12} from its rat stomach extract complex with equal ease but attempts to demonstrate equivalent release of radio-

TABLE VI. Releasing Activity of Human Organ Extracts.

Extract	Vit. $B_{12}Co^{60}$ released from NHGJ	Vit. B_{12} content
	(%)	(mμg/ml)
Liver	46.0	70.0
Lung	11.0	8.5
Heart	4.7	5.0
Skeletal muscle	2.4	9.0
Pancreas	9.6	12.0
Kidney	8.6	20.0
Brain	3.6	12.0
Spleen	.8	5.0
Small intestine	4.4	11.0

NHGJ = Normal human gastric juice.

active vit B_{12} from hog intrinsic factor complex by rat, beef or hog intestinal extracts have been unsuccessful. Exchange between unbound vit B_{12} and that bound to hog or rat intrinsic factor preparations was studied in the releasing factor assay system. From 2 to 25 mμg of unbound vit B_{12} was equilibrated with 25 mμg of radioactive B_{12} complexed with either hog or rat intrinsic factor preparations. Examination of Fig. 2 reveals that vit B_{12} bound to rat stomach extract undergoes decidedly more exchange than that bound to hog intrinsic factor. Approximately 70% of complete exchange was found with vit B_{12}-rat stomach complex and about 7% with vit B_{12} bound to hog intrinsic factor. The latter finding is in agreement with previous results with hog intrinsic factor at 4°C(12). This could explain our inability to demonstrate release of vit B_{12} from hog intrinsic factor by intestinal segment extracts.

Discussion. From results obtained in this investigation, one must conclude that exchange takes place between vit B_{12} bound to rat stomach extract and endogenous vit B_{12} contained in intestinal segment extracts or organ extracts. Of prime importance is whether exchange of vit B_{12} accounts for all or a major portion of releasing factor activity displayed by intestinal segment extracts and human organ extracts. The fact that releasing factor activity is dialyzable suggests that liberation of bound vitamin B_{12} by intestinal extracts is not an enzymatic process. We have investigated the partial inactivation obtained by heating proximal and mid-ileal extracts(7) and find that inactivation previously observed was probably due to occlusion of endogenous vit B_{12} in the coagulum caused by heat denaturation of the protein. Agitation of the heat treated extracts before assay greatly reduced the apparent inactivation of both segment extracts. The only evidence that endogenous vit B_{12} may not be solely responsible for release of vit B_{12} was the rather poor correlation between endogenous vit B_{12} lost during exhaustive dialysis and loss of releasing factor activity and the fact that endogenous vit B_{12} does not appear to account for all the activity displayed by segment 1.

Species specificity of the releasing factor activity obtained from rat and human intestinal extracts was previously reported(13). Using identical methodology we have been unsuccessful in again preparing material demonstrating a species specific ability to release vit B_{12} from intrinsic factor. However, a small amount of frozen intestinal extract made 4 and 5 years ago still demonstrates species specificity. At present we have no explanation for this.

It should be noted that a complex equilibrium system exists between bound and unbound vit B_{12} in a mixture of rat stomach and intestinal segment extract or normal human gastric juice and human organ extracts. Both contain endogenous vit B_{12} which is not removed by exhaustive dialysis, as well as dialyzable endogenous vit B_{12}. When radioactive vit B_{12} is added, the system becomes more complex. This could explain the variability of results obtained with different batches of rat stomach and intestinal segment extracts both before and after dialysis of the latter. For this reason, it is difficult to conclude whether endogenous vit B_{12} is solely responsible for the observed release of radioactive vit B_{12}. However, most of the evidence presented here suggests that in the assay system used the equilibration of endogenous vit B_{12} with radioactive vit B_{12} bound to rat or human intrinsic factor concentrates can account for substantially all the activity displayed by the rat small intestine or human organ extracts.

In view of the fact that vit B_{12} is not bound to intrinsic factor in the serum but to a material with entirely different properties, a system for releasing vit B_{12} from intrinsic factor probably exists. It is unknown whether this system involves a "releasing" enzyme or is inherent in the nature of the vit B_{12}-intrinsic factor bond. The site of this release of vit B_{12} from intrinsic factor is also unknown.

Summary. Experiments were performed for the purpose of grossly identifying the substance responsible for the release, by rat intestinal segment extracts and human organ extracts, of vitamin B_{12} bound to rat stomach extract or normal human gastric juice. It has

been found that the active principle in the proximal area and mid-ileum of the rat small intestine is dialyzable and that its distribution parallels closely that of endogenous vitamin B_{12}. Non-radioactive vitamin B_{12} has been found to be very active in the release of radioactive vitamin B_{12} bound to rat or human intrinsic factor concentrate. This appears to be due to its ability to equilibrate with the bound form. In a complex equilibrium system, endogenous vitamin B_{12} appears to account for substantially all of the activity displayed by the proximal area and mid-ileum of the rat small intestine, and by mucosa homogenates from beef small intestine and various human organ extracts. Vitamin B_{12} bound to hog intrinsic factor on the other hand shows little tendency to equilibrate with unbound vitamin B_{12}.

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Hepatic Clearance of Indocyanine Green in the Beagle. (30241)

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Following its introduction for the measurement of blood flow(1) indocyanine green dye (ICG) was found to be useful for assessment of hepatic activity in dogs(2). Wheeler *et al* (3) found an exponential plasma disappearance rate of 6.6 and 13.3% per minute in 2 dogs; Rapaport and coworkers(4) reported a rate of 18.1% per minute in anesthetized dogs. Other investigators have found plasma disappearance rates of 7.6% per minute, 6.9% per minute and 8.9% per minute (5,2,6).

Because the beagle dog is extensively used as an experimental animal, the purpose of this study was to investigate the rate of ICG clearance from plasma in this breed of dog, and to compare it with values obtained in mongrel dogs.

Methods. ICG clearance was determined by the method of Ketterer *et al*(2) in 24 purebred beagle dogs between one and two years old, maintained within our colony for at least 4 months and 12 mongrel dogs housed for less than one week. Liver function was judged to be normal in all animals based on alkaline phosphatase (AP) and serum glutamic oxalacetic transaminase values (SGOT) determined by standard methods (7,8).

All dogs were fasted overnight. An aqueous solution of ICG* (2.5 mg/ml) was given intravenously, *via* a brachial vein, at a dosage schedule of 0.5 mg/kg. Two or 3 heparinized blood samples were taken from the jugular

* Indocyanine green (Cardio Green), Hynson Westcott & Dunning, Baltimore.