sustained maximum effort(10). Moreover, prolonged testing periods make it virtually impossible to retain cell cultures with similar susceptibility characteristics since these are subject to change with succeeding generations of cells.

Diagnostic virus serology by MTC methods is made more feasible and relatively inexpensive for even the smallest tissue culture laboratory. The reduction of serum requirements is appropriate for neutralizing antibody studies of children or infants where only small amounts are usually available.

Finally, it is possible that many if not all conventional tissue culture techniques can be applied to the MTC method with its subsequent economy and ease of replication so as to greatly enhance virus research as well as diagnostic efforts.

Summary. A simplified method for virus-tissue culture practices, accomplished in microtitration plates reduces time, materials, and effort, yet agrees well with results obtained by conventional tissue culture tube methods. Neutralization tests for poliovirus Type III performed by both methods indicated a high degree of compatibility.

Addendum. Since the completion of this study, disposable MTC plates have been produced by the manufacturer.

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Release of Vitamin B_{12} and Cobamide Coenzyme from Rat Intrinsic Factor by Rat Intestinal Extracts. (28326)

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Studies by Ungley showed that the intestinal juice of a pernicious anemia patient was able to release Vitamin B_{12} from a Vitamin B_{12}-normal gastric juice complex(1). Ungley proposed the existence of an enzyme in the small intestine capable of splitting Vit. B_{12} from an intrinsic factor complex. Cooper and Castle confirmed these observations using rat intestinal extract and rat intrinsic factor concentrate(2). The active principle in the intestinal extracts, capable of releasing Vit. B_{12} from its intrinsic factor complex, has been termed releasing factor. Recently, the physiological role of releasing factor has been questioned by Herbert *et al.* (3), who reported that releasing factor activity was restricted to the proximal 10% of the rat small intestine and was not present in the mid-gut where Vit. B_{12} absorption takes place. We have examined the location of releasing factor activity in the rat small intestine and contrary to the results of Herbert *et al.* find it to be present in substantial amounts in the mid-gut as well as in the proximal area. These findings are consistent with the postulated function of releasing fac-

tor in intestinal absorption of Vit. B_{12}.

Materials and methods. Protein determination. The improved Folin-phenol procedure described by Lowry *et al.* was used(4).

Radioactive materials. Vitamin B_{12} labeled with Co^{60} was purchased from Merck and Co. The labeled cobamide coenzyme (5,6-dimethylbenzimidazolyl cobamide coenzyme) was a gift of Dr. David Perlman, Squibb Institute for Medical Research.

Measurement of radioactivity was performed in a sodium iodide or plastic well scintillator previously described(5).

Preparation of rat intrinsic factor (stomach extract) and releasing factor (intestinal extract). Adult male rats (Carworth CFE) were used throughout the study. The rats were sacrificed by a blow on the head and their stomachs and small intestines quickly removed. These organs were rinsed inside and outside with chilled saline or phosphate buffer, pH 7.6, 0.075 M. The small intestine was cut into 10 segments of equal length. The segments from 10 rats were pooled before further processing which was carried out at 4°C. The pooled rat stomachs and pooled segments of the small intestine were each homogenized in a Waring blendor with cold phosphate buffer. Usually, 50 ml of buffer was used with each pool of 10 stomachs and 20 ml of buffer was used for each pool of 10 intestinal segments. The homogenates were centrifuged at 15,000 × g for 15 min. The residue was discarded and the supernatants frozen until used.

Measurement of releasing factor activity. Sufficient labeled Vit. B_{12} was added to the rat intrinsic factor just to saturate the Vit. B_{12} binding capacity of the preparation. The stated amounts of the Vit. B_{12}-intrinsic factor complex were mixed with the stated amounts of the small intestinal extracts and sufficient buffer added to give a total volume of 3 ml. This solution was placed in a piece of 20/32 dialysis tubing (about 5½ inches long) which was tied at one end. A 5-inch length of No. 4 glass rod was placed in the bag and the other end of the bag was closed. The dialysis bag containing the reaction mixture and the glass rod was placed in a 16 × 150 mm test tube which contained 9 ml of buffer. The test tube was tightly stoppered and placed horizontally in a circular rotator (10 r.p.m.) for 6 hours at 37°C in the dark. At the end of 6 hours the dialysis tubing was removed from the test tube and the outside of the tubing rinsed with buffer. These rinsings were combined with the 9 ml of solution on the outside of the dialysis tubing and counted for radioactivity.

TABLE I. Protein Concentration and Vitamin B_{12} Binding Capacity of Rat Stomach Extract and Rat Intestinal Extract (Pool of 10 Rats).

		Protein concentration, mg/ml	Vit. B_{12} binding, μg/ml
Stomach		17	.30
Portion of small intestine			
(Proximal end)	1	27	.00
	2	20	.01
	3	20	.01
	4	20	.01
	5	18	.01
	6	17	.01
	7	16	.02
	8	17	.01
	9	17	.02
(Ileal end)	10	18	.02

Results. The protein concentration and Vit. B_{12} binding capacity of the stomach extract and the rat intestinal extracts of a typical preparation are shown in Table I. Almost identical results were obtained with 3 additional preparations. Of primary interest is the negligible Vit. B_{12} binding capacity of the small intestine and the higher protein content of the extract from the proximal end compared with other portions of the small intestine. The protein concentration of the proximal end was approximately 33% higher than the average of the other 9 segments. This varied slightly from preparation to preparation but it was never less han 30% or higher than 50% of the average of the other 9 segments.

The results of a typical experiment using 1.0 ml of intestinal extract as a source of releasing factor activity are shown in Table II. The same amount of substrate (Vit. B_{12}-rat stomach extract was used with each of the intestinal extracts. It is evident that 2 peaks of releasing factor activity were ob-

TABLE II. Releasing Factor Activity of Extracts Prepared from 10 Equal Segments of Rat Small Intestine. The Vit. B_{12}-rat intrinsic factor complex was prepared by mixing 5 ml of rat stomach extract (Table I) with 1 μg (1 ml) of Vit. $B_{12}Co^{60}$. Amount of substrate used in each of the experiments below was 0.2 ml, which contained 2.8 mg protein and 33 mμg Vit. B_{12}.

	Portion of small intestine	Vit. B_{12} released*	
		mμg	%
1	Proximal end (1.0 ml)	13.7	41.5
2	"	3.6	10.9
3	"	4.8	14.5
4	"	7.0	21.2
5	"	8.0	24.2
6	"	7.6	23.0
7	"	5.9	17.9
8	"	4.9	14.8
9	"	3.7	11.2
10	Ileal end "	3.1	9.4

* Corrected for control (Vit. B_{12}-rat intrinsic factor without intestinal extract).

served, namely the proximal end (segment 1) and the mid-gut (segments 4, 5 and 6). The higher releasing factor activity observed in the proximal end could be accounted for only in part by its higher protein content. In most of our experiments about twice as much releasing factor activity was observed with the proximal end as was seen in segments 4, 5 or 6.

The results of experiments in which the amount of intestinal extracts of segments 1 and 5 were varied are shown in Fig. 1. A linear relationship between activity and concentration was observed with as much as 0.5 ml of intestinal extract. This amount was used in various inhibition studies discussed below.

TABLE III. Effect of Various Metabolic Inhibitors and Heat on Releasing Factor Activity of Segments 1 and 5. The inhibitors were present at a final concentration of 10^{-2}M. Conditions of experiment were the same as described under *Methods* and Table II. Amount of substrate (Vit. B_{12}-intrinsic factor complex) used was 0.2 ml and amount of intestinal extract used was 0.5 ml. Values are expressed as percent inhibition compared to control (without inhibitors).

Treatment	% inhibition	
	Seg 1	Seg 5
2,4-Dinitrophenol	0	12.3
Sodium fluoride	1.2	8.3
" arsenite	2.7	0
Potassium cyanide	3.7	0
Heated (15 min)	47.7	77.8

The effect of various metabolic inhibitors on the releasing factor activity of segments 1 and 5 is shown in Table III. The almost negligible inhibition observed with inhibitors at a final concentration as high as 10^{-2} M suggests that the release of Vit. B_{12} was not due to an energy-requiring metabolic reaction.

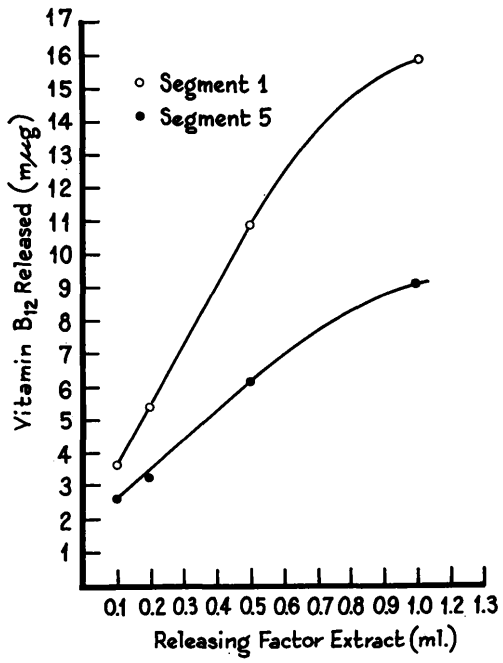

FIG. 1. Effect of concentration of releasing factor on amount of Vit. B_{12} released. Conditions of experiment identical to those described in Table II.

The effect of heating on the releasing factor activity is also shown in Table III. The intestinal extracts were heated in a boiling water bath for 15 minutes before mixing with the substrate. It is of interest that the activity of segment 5 was somewhat more heat labile than that of segment 1.

Latner *et al.*(6) have suggested that Vit. B_{12} is probably converted to its coenzyme form during gastrointestinal absorption. It was of interest therefore, to determine the distribution of releasing factor activity using 5,6-dimethylbenzimidazolyl cobamide coenzyme instead of cyanocobalamin. The results of such an experiment are shown in Table IV. It is evident that the same distribution of releasing factor activity was ob-

TABLE IV. Releasing Factor Activity of Small Intestine Using Coenzyme B_{12} Intrinsic Factor Complex as Substrate. Three ml of rat stomach extract was mixed with 0.6 μg (0.5 ml) of Co^{60} labeled 5,6-dimethylbenzimidazoyl cobamide coenzyme. This mixture served as the substrate. The amount of substrate used in each incubation was 0.2 ml which contained 34 mμg of the coenzyme and 2.9 mg protein. Amount of releasing factor used was 0.5 ml in each incubation. To compare this with the release of cyanocobalamin, 0.5 ml of intestinal extract of segment 1 and 5 were run simultaneously and the results are shown.

Portion of small intestine (segment No.)	Coenzyme B_{12} released	
	mμg	%
1 Proximal	10.4	30.7
2	3.3	9.6
3	3.8	11.2
4	3.8	11.2
5	4.7	13.8
6	4.7	13.8
7	4.5	13.3
8	3.6	10.5
9	3.2	9.4
10 Ileal end	2.9	8.5
	Vit. B_{12} released	
1	11.9	36.0
5	4.8	14.4

served with the coenzyme bound to rat intrinsic factor as was found with cyanocobalamin. Segments 1 and 5 using Vit. B_{12} (cyanocobalamin) were run simultaneously in order to compare the results with those obtained with the coenzyme. There was excellent quantitative agreement.

Discussion. Booth *et al.*(7) have clearly demonstrated that the middle portion of the small intestine is the site of maximum Vit. B_{12} absorption in the rat. More specifically, these investigators showed that when the small intestine was divided into 10 equal segments, maximum absorption was observed between the 4th and 8th segments. Herbert *et al.*(3) questioned whether releasing factor had a physiologic role in Vit. B_{12} absorption because they were able to demonstrate releasing factor activity in the proximal 10% of the rat small intestine but not near the site of Vit. B_{12} absorption. Our experiments also demonstrate releasing factor activity in the proximal 10% of the small intestine but we have shown another peak of activity in segments 4-8. Our latter finding is quite consistent with observations on the site of Vit. B_{12} absorption in the rat.

The failure of Herbert *et al.*(3) to find releasing factor activity in the mid-gut could possibly be explained by differences in technique used by the above investigators and ourselves. We prepared both stomach and intestinal extracts by centrifuging the respective homogenates at 15,000 $\times$ g at 4°C. Herbert *et al.* used the supernatants from centrifuging at 3,000 $\times$ g. The temperature they used was not specified. Our preliminary experiments indicated that considerably more activity was obtained if the material was prepared in the cold and centrifuged at higher speeds to obtain clearer supernatants.

The finding of 2 peaks of releasing factor activity in the small intestine is unique. Attempts to investigate by kinetic means whether the active substance (possibly an enzyme) present in the proximal end is identical to that in the mid-gut have been inconclusive. To study the reaction kinetically, it would be necessary to be certain that the studies were performed within the period of constant velocity of the reaction. We have not yet investigated methods of stopping the reaction so that it would be possible to measure reaction velocities. The effect of temperature and pH on the quantitative activity of releasing factor would also be helpful to determine similarities or differences in the activity of segment 1 and segments 4-8. Throughout our present study we have used pH 7.6 which was found by Doscherholmen (8) to be the pH optimum for whole rat intestinal extract. Suggestions that the substance in segment 1 may differ from that of segment 5 comes from small differences obtained with metabolic inhibitors and from heat inactivation studies. This is by no means conclusive evidence.

The distribution of "releasing factor" activity in the rat intestine invites the postulation of 2 distinct areas of the small intestine which may be involved in the overall mechanism of Vit. B_{12} absorption. The activity in segments 4-8 would appear to be releasing factor activity associated with the removal of Vit. B_{12} from intrinsic factor at the intestinal wall. The activity of segment 1 could be thought of as the "Vit. B_{12} trans-

fer" factor previously proposed(9). One could postulate that the proximity of segment 1 to the area of intrinsic factor secretion would make it the logical site of transfer of Vit. B_{12} from dietary proteins to intrinsic factor.

It is of interest that the same anatomical distribution of releasing factor activity in the rat small intestine was observed with 5,6-dimethylbenzimidazolyl coenzyme B_{12} as was found with Vit. B_{12} bound to intrinsic factor. Good quantitative agreement was also found with both cobalamins and the intestinal extracts of segments 1 and 5. This is consistent with the observation that the coenzyme is as well absorbed from the gastrointestinal tract as cyanocobalamin(10).

Summary. The ability of rat intestinal extracts to release Vit. $B_{12}Co^{60}$ bound to rat intrinsic factor extract was studied using equilibrium dialysis. The small intestines of adult rats were divided into 10 equal segments and extracts of each segment were prepared. Releasing factor activity was detected in all the segments but 2 peaks of activity were found in the proximal area (segment 1) and the mid-portion of the small intestine (segments 4, 5 and 6). Releasing factor activity of the proximal and mid-portion was partially inactivated by heat. Sodium fluoride, sodium arsenite, potassium cyanide or 2,4-dinitrophenol at 10^{-2} M did not markedly inhibit the releasing factor activity. The same anatomical distribution of releasing factor activity was observed with 5,6-dimethylbenzimidazolyl cobamide coenzyme bound to rat intrinsic factor. The finding of releasing factor activity in the midgut is consistent with observations on the site of absorption of Vit. B_{12} in the rat.

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Conjugation of C^{14}-Diethylstilbestrol by Chicken Liver *in vitro*.* (28327)

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The role of the liver in the inactivation of estrogens has been extensively investigated as reviewed up to 1950 by Paschkis and Rakoff(1). Zondek *et al.*(2) demonstrated that diethylstilbestrol (DES) could be inactivated *in vitro* by rat liver pulp but to a lesser extent than estrone while prior treatment with DES *in vivo* increased the inactivating influence of the liver. The DES metabolites resulting from liver action were extracted for assay in spayed female mice. Zimmerberg(3) determined that, for rat liver, conjugation was the preferred means of inactivation, oxidation occurring only when the conjugating capacity of the tissue was ex-

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