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EXCHANGE BETWEEN FREE AND GASTRIC JUICE-BOUND CYANOCOBALAMIN *

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The relation between intrinsic factor (IF) and the vitamin B_{12}-binding activity of gastric juice is controversial. There is considerable evidence that IF either binds vitamin B_{12} or is intimately associated with a binding substance (2), but the importance of binding in IF function is not clear (3). It is apparent that binding of vitamin B_{12} is not the sole basis for IF action, since there are many substances that bind the vitamin but lack IF function (4, 5). It has been suggested that absorption of physiological quantities of vitamin B_{12} requires that the vitamin be bound to IF when it reaches absorption sites (6, 7). Under these conditions, the quantitative estimation of vitamin B_{12} absorption derived from isotope studies would depend upon the amount of radioactive cyanocobalamin (radioB_{12}) bound to IF at the time of absorption. Biliary excretion of vitamin B_{12} has been documented (8, 9) and an enterohepatic circulation of the vitamin postulated (8). If exchange took place in the intestinal lumen between this unlabeled vitamin B_{12} and IF-bound radioB_{12}, then measurements of radioactivity would underestimate the total amount of vitamin B_{12} absorbed. Previous investigations have not been directly concerned with the possibility that such an exchange might occur.

This report describes studies of gastric juice binding and absorption of radioB_{12} in which Co^{60}-labeled cyanocobalamin ($Co^{60}B_{12}$) and Co^{57}-labeled cyanocobalamin ($Co^{57}B_{12}$) were used simultaneously. A significant exchange between free and gastric juice-bound cyanocobalamin was observed *in vitro*. Absorption tests carried out in pernicious anemia patients and in gastrectomized rats suggested that IF was involved in this exchange. Exchange also occurred between gastric juice-bound radioB_{12} and unlabeled vitamin B_{12} present in the intestinal juice and tissues of rats.

METHODS

Collection of gastric juice. Human gastric juice was obtained after the administration of histamine (10) to fasting hospitalized patients. Care was taken to prevent contamination with saliva, and specimens containing bile or blood were discarded. The juice obtained during each 15-minute period was immediately titrated electrometrically to pH 10 with 10% NaOH in order to inactivate pepsin (11). After 20 minutes, the pH was adjusted to 7 with 1 N HCl and the juice was then filtered through coarse filter paper. During collection and neutralization, the individual specimens were kept chilled. The 3 or 4 specimens obtained from each patient were pooled and stored in small samples at −20° C.

Gastric juice was collected from fasting albino rats by ligating the pylorus and instilling 1 ml of 10% Na_2CO_3 into the stomach (11). Five hours later, the stomach was removed and its contents were emptied into a flask. A volume of 3 to 12 ml of juice at pH 8.1 to 9.8 was obtained from each rat. Juice from several rats was pooled, neutralized to pH 7.0, filtered, and stored at −20° C.

Measurement of radioactivity. Samples containing $Co^{57}B_{12}$ and $Co^{60}B_{12}$ were counted in well-type scintillation counters. Samples of small volume were measured by means of a counter containing a 1¾-inch sodium iodide crystal and equipped with an automatic sample-changing device. Samples of large volume were counted in an instrument containing a 2¾-inch crystal and accommodating 25-ml vials. Both instruments were equipped with spectrometers. Because of a wide difference in their gamma spectra, Co^{60} and Co^{57} were easily resolved in mixtures of the two. Optimal window and voltage settings for each isotope were obtained in order to give maximal efficiencies and minimal cross-counting. For mixtures of the isotopes the following calculations were applied: Co^{60} *counts* $= [N_{60} - R_2 (N_{57})]/(1 - R_1R_2)$ and Co^{57} *counts* $= [N_{57} - R_1 (N_{60})]/(1 - R_1 R_2)$, where N_{60} equals counts obtained for mixed samples at optimal Co^{60} settings,

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N_{57} equals counts obtained for mixed samples at optimal Co^{57} settings, R_1 equals N_{57}/N^{60} for pure Co^{60}, and R_2 equals N_{60}/N_{57} for pure Co^{57}. Since R_1 was 0.04 to 0.09 and R_2 was 0.003 to 0.005, the denominator $(1 - R_1R_2)$ was considered to equal unity.

Enough counts were obtained at each setting to reduce counting error to less than 2%. When standard samples containing various mixtures of the two isotopes were compared with samples containing a single isotope, the results agreed to within 2%.

Absorption tests. The absorption of 0.25 μg of radio-B_{12} was tested in fasting, hospitalized patients by means of the Schilling test (12). A "flushing" dose of 1,000 μg of cyanocobalamin was administered intramuscularly 2 hours after radioB_{12} had been given orally; urine was then collected for 24 hours. A 25-ml sample of urine was counted. The pernicious anemia patients tested had been shown to have histamine-fast achlorhydria, megaloblastic anemia, low serum vitamin B_{12} levels, and a reticulocyte response to injected cyanocobalamin.

In rats, absorption was studied by a fecal excretion test previously described in detail (13). Stools were collected for 7 days after 0.01 μg of radioB_{12} had been administered into the stomach through a fine polyethylene tube.

Separation of free and bound cyanocobalamin by dialysis, starch gel electrophoresis, and dextran gel filtration. Dialysis was used to separate free from gastric juice-bound radioB_{12}, since the latter is unable to pass through a cellophane membrane (11). Duplicate 2-ml samples were placed in Visking cellophane bags and dialyzed with constant stirring against 3 L of 0.15 M NaCl for 72 hours at 6° C. The dialysate was replaced daily. The bags were then removed and their contents were assayed for radioactivity.

Electrophoretic separation of free and gastric juice-bound radioB_{12} was accomplished by the vertical starch gel technique described by Smithies (14). Electrophoresis was carried out for 15 hours at room temperature with 0.023 M borate buffer at pH 8.6. In this system, as has been shown previously (15), free radioB_{12} migrates cathodally, whereas radioB_{12} bound to normal gastric juice migrates anodally. Since concentration of gastric juice was avoided in these experiments, no amido black-staining material was detected after electrophoresis. Therefore, normal human serum was placed in each of the outermost slots of the gel to serve as a reference for the migration of radioactivity. After electrophoresis, the two areas of gel containing serum were sliced and stained with amido black 10B. Under the conditions described, beta$_1$-globulin (transferrin C) moved anodally 5.2 to 6.4 cm. RadioB_{12} bound to human or rat gastric juice had the same mobility as the postalbumin region of normal human serum. Recovery of radiocativity applied to starch gels varied from 85 to 97%.

The use of dextran gel (Sephadex) columns to separate free and bound radioB_{12} has not been previously described in detail, although Kakei and Glass (16) have published an abstract concerned with dextran gel filtration as a means of measuring the binding of cyanocobalamin. The dextran gel used in this study (Sephadex G-50) excludes substances with molecular weights greater than 8,000 to 10,000. Thus, free radioB_{12} (molecular weight 1,355) mixes in the intragel water phase and is not completely eluted until a volume of eluant equal to the total column volume is applied. On the other hand, radioB_{12} bound to the substances of gastric juice with large molecular weights does not mix in the intragel water phase and is therefore eluted by a volume of eluant equal to the volume of the external water.

In the present study, 2-ml samples of free, or bound radioB_{12}, or both were applied to 15 × 1.6-cm columns of Sephadex G-50. The columns were then eluted with 0.15 M NaCl at a rate of 0.3 to 0.8 ml per minute. The eluate was collected with an automatic fraction collector in 3.0 to 5.0-ml samples. Satisfactory separation of free and bound radioB_{12} was regularly accomplished within 90 minutes and was reproducible (Figure 1). In 45 experiments, the recovery of radioactivity applied to the columns was 98.9 ± 2.4 (SD)%. Radioactivity eluted from the column in the "bound peak" (peak I) was non-dialyzable and had the same electrophoretic mobility as gastric juice-bound radioB_{12}. Radioactivity eluted in the "free peak" (peak II) was dialyzable and migrated with the same electrophoretic mobility as free radioB_{12}. When increasing amounts of radioB_{12} were added to a constant amount of gastric juice, the quantity of radioactivity appearing in peak I remained constant while that in peak II increased.

RadioB_{12} was added in excess to each of nine specimens of neutralized human gastric juice, and the binding capacity of these specimens was determined both by dialysis and by dextran gel filtration. The binding capacities as measured by both methods agreed in each case within 3%.

Two separate Schilling tests were performed in a patient with pernicious anemia. When 0.25 μg of radioB_{12} eluted in peak I was administered orally, 18.9% of the radioactivity was excreted in the urine. In the second test, only 2.5% of 0.25 μg of radioB_{12} obtained from the column in peak II was excreted.

Measurement of endogenous vitamin B_{12}[1] activity. Fasting female albino rats weighing 150 to 250 g were sacrificed by a blow on the head. The intestine was quickly ligated and divided at the pylorus and at the ileocecal valve. The entire small intestine was then removed, and its contents were gently flushed into a graduated tube with 5 ml of chilled Krebs-Ringer-bicarbonate solution (KRB) (17). Everted sacs were made from 10-cm segments of mid-intestine and were incubated in 5 ml of KRB according to the method of Wilson and Wiseman (18). Liver and segments of mid-intestine were homogenized in Potter-Elvehjem glass homogenizers in 5 ml of chilled KRB. These homogenates were then washed with additional chilled KRB, centrifuged,

[1] The term "vitamin B_{12}" is used here in a generic sense and refers to substances that promote growth of *Euglena gracilis* in the bioassay.

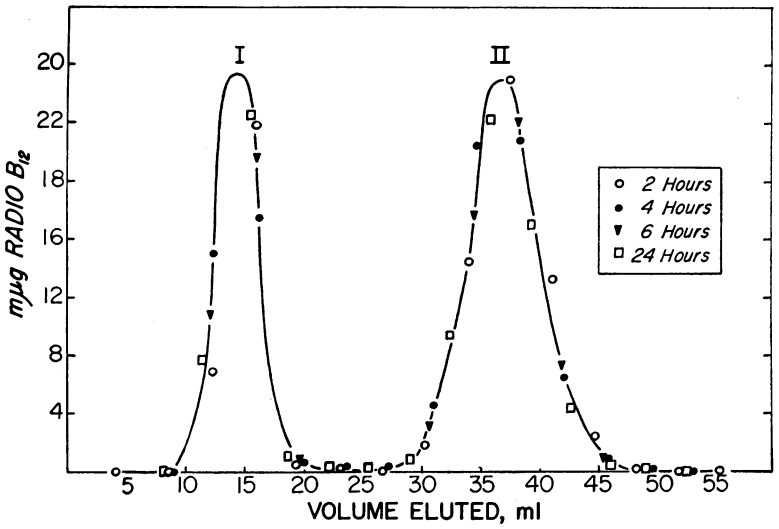

FIG. 1. SEPARATION OF FREE AND HUMAN GASTRIC JUICE-BOUND RADIOB_{12} BY DEXTRAN GEL FILTRATION. An excess of $Co^{60}B_{12}$ was added to normal human gastric juice, and the mixture was incubated at 37° C. After 2, 4, 6, and 24 hours, a 2-ml sample of the mixture was applied to the same dextran gel column and eluted with 0.15 M NaCl. Elution rates and collection volumes were varied slightly to demonstrate the reproducibility of elution patterns. Only peak I radioactivity was nondialyzable. Incubation at 37° C did not alter the binding capacity of gastric juice.

and finally resuspended in 5 ml of KRB. Everted intestinal sacs, intestinal homogenates, and liver homogenates were incubated for 1 hour at 37° C with shaking in an atmosphere saturated with 95% O_2 and 5% CO_2. After incubation, the everted sacs were removed from the flasks, dried in an oven, and weighed. Liver and intestinal homogenates were separated from the supernatant fluid by centrifugation at 25,000 × *g* for 5 minutes, dried, and weighed. Total vitamin B_{12}-like activity of intestinal juice and of KRB that had been incubated with everted intestinal sacs, intestinal homogenates, and liver homogenates was measured by *Euglena gracilis* assay (19).

RESULTS

Exchange between free and gastric juice-bound radioB_{12} in vitro. Bound radioB_{12} was prepared by adding $Co^{60}B_{12}$ to neutralized rat or human gastric juice at room temperature. The amounts of $Co^{60}B_{12}$ added were approximately twice those necessary to saturate the binding capacities of the gastric juice. One hour later the mixture was subjected to dialysis for 72 hours at 6° C. The bound $Co^{60}B_{12}$ remaining in the bag was then removed and incubated at pH 6.8 to 7.0 for varying periods with an equal quantity of free $Co^{57}B_{12}$. After incubation, 2-ml samples of the mixture were dialyzed for 72 hours at 6° C. The amounts of $Co^{57}B_{12}$ and $Co^{60}B_{12}$ remaining in the bag were then determined. In control experiments, bound $Co^{60}B_{12}$ was mixed with 0.15 M NaCl, incubated for varying periods, and then dialyzed for 72 hours. Exposure of human or rat gastric juice to repeated and prolonged dialysis at 37° C did not result in a significant decrease in binding capacity or loss of IF activity of the gastric juice. In 14 experiments, the binding capacity after dialysis at 37° C was 91 to 102% of that observed when the same specimens were dialyzed at 6° C. Retention of IF activity after prolonged dialysis was demonstrated in 15 patients with pernicious anemia and in 2 gastrectomized rats.

As illustrated in Table I, it was regularly observed that a significant quantity of the initially free $Co^{57}B_{12}$ became bound (nondialyzable) while a similar amount of the $Co^{60}B_{12}$ disappeared from the dialysis bag. The longer the mixtures were incubated before dialysis, the greater was this exchange between the two isotopes, but the total amount of bound radioB_{12} did not change significantly. In six other dialysis experiments, the protocol was similar, but the amounts of radioB_{12}

TABLE I

Demonstration of exchange between free and bound radioactive cyanobalamin (radioB_{12}) by dialysis

Time	RadioB12 remaining in dialysis bags			
	Mixture of free Co^{57} B_{12} and bound Co^{60} B_{12}*			
	Co^{57} B_{12}	Co^{60} B_{12}	Total radioB_{12}	Bound Co^{60} B_{12}* alone
	mμg	mμg	mμg	mμg
Before dialysis	12.7	12.4	25.1	12.4
After dialysis† following incubation for: 2 hours	3.5	8.9	12.4	12.1
4 hours	4.4	7.7	12.1	11.8
8 hours	5.3	6.9	12.2	11.9
24 hours	5.8	6.7	12.5	11.9

* Bound to neutralized human gastric juice.

† After incubation at 37°C, the mixture of Co^{57} B_{12} and Co^{60} B_{12} or the bound Co^{60} B_{12} alone was dialyzed at 6° C for 72 hours.

were varied and the isotopic forms of radioB_{12} were alternated. Exchange was demonstrated in all experiments, and the percentage of theoretical exchange in all periods never varied more than 14%.

Exchange between the two isotopes was expressed as the percentage of theoretically complete exchange. Thus, if the mixture initially contained exactly equal quantities of bound $Co^{60}B_{12}$ and free $Co^{57}B_{12}$, exchange would be complete when one-half of the radioB_{12} remaining in the dialysis bag consisted of the initially free $Co^{57}B_{12}$. In all experiments, therefore, the amount of exchange that occurred was calculated by dividing the observed ratio of the two isotopes by the ratio expected for complete exchange. Experiments were carried out in which mixtures were incubated at pH 6.8 to 7.0 at 37°, 22°, and 6° C. As Figure 2 shows, the rate of exchange between free and bound radioB_{12} increased as the temperature increased. The rate of exchange was similar for both rat and human gastric juice.

Mixtures containing varying proportions of $Co^{57}B_{12}$ and $Co^{60}B_{12}$ were added to either rat or human gastric juice, and after incubation for 1 hour at room temperature, dialysis was carried out for 72 hours. The relative amounts of the two isotopes that became nondialyzable were the same as the relative amounts present before dialysis. Therefore, one isotope did not appear to be more readily bound by gastric juice than the other. When gastric juice-bound radioB_{12} was incubated at 37° C with 10^{-4} M cobalt sulfate for 24 hours and then dialyzed, the amount of bound radioactivity did not decrease. Thus the observed exchange between free and bound radioB_{12} apparently involved the entire cyanocobalamin molecules rather than the cobalt isotopes per se.

In vitro exchange between free and gastric juice-bound radioB_{12} could also be demonstrated by

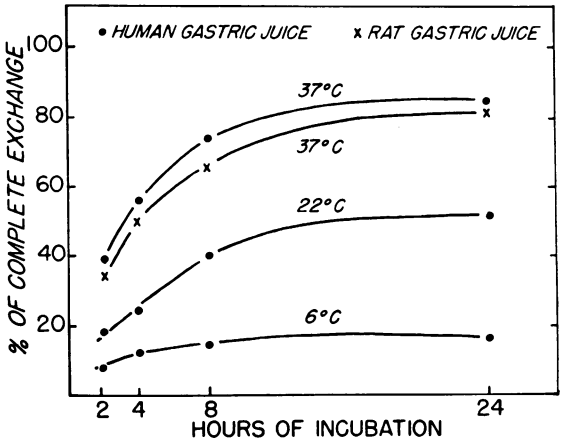

FIG. 2. EFFECT OF TEMPERATURE ON EXCHANGE BETWEEN FREE AND BOUND RADIOB_{12}. Mixtures of free $Co^{57}B_{12}$ and gastric juice saturated with $Co^{60}B_{12}$ were incubated at various temperatures. At 2, 4, 8, and 24 hours, samples of the mixtures were dialyzed in cellophane bags for 72 hours at 6° C, and the amounts of nondialyzable Co^{57} and Co^{60} were then determined. The observed exchange between free and bound radioB_{12} was expressed as the percentage of theoretically complete exchange as defined in the test. In two other experiments, the protocol was similar except that the amounts of radioB_{12} used and the times of incubation were different. In all experiments, rate of exchange increased with increasing temperature, and percentage of theoretical exchange at 24 hours always agreed within 10%.

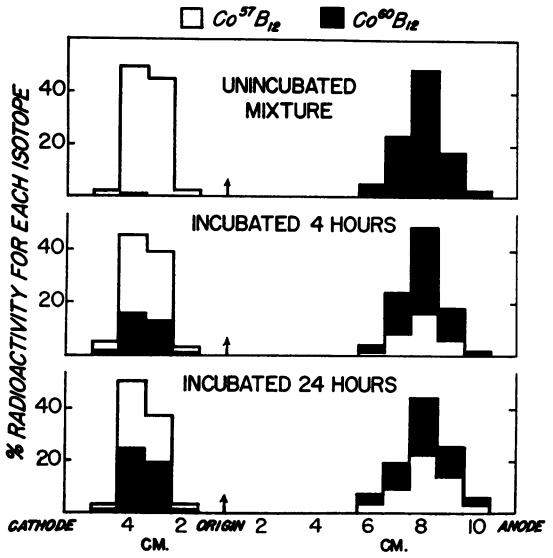

FIG. 3. DEMONSTRATION OF EXCHANGE BETWEEN FREE AND GASTRIC JUICE-BOUND RADIOB_{12} BY STARCH GEL ELECTROPHORESIS. A mixture of equal quantities of $Co^{60}B_{12}$ bound to human gastric juice and free $Co^{57}B_{12}$ was subjected to starch gel electrophoresis. Bound $Co^{60}B_{12}$ migrated anodally while free $Co^{57}B_{12}$ migrated cathodally. When the mixture was incubated at 37° C for 4 and 24 hours, an exchange between $Co^{57}B_{12}$ and $Co^{60}B_{12}$ was noted. The total amount of radioB_{12} in each of the peaks remained constant.

starch gel electrophoresis. When free $Co^{57}B_{12}$ and bound $Co^{60}B_{12}$ were mixed and immediately subjected to electrophoresis, separation of the two isotopes on starch gel was virtually complete, as Figure 3 shows. When, however, this mixture was incubated at pH 6.8 to 7.0 at 37° C, significant quantities of the initially free $Co^{57}B_{12}$ migrated anodally while equal amounts of $Co^{60}B_{12}$ moved cathodally. During these experiments, the total amounts of free and bound radioB_{12} remained constant.

Finally, mixtures of free and gastric juice-bound radioB_{12} were subjected to dextran gel filtration after incubation at 37° C. Exchange between bound and free radioB_{12} was again demonstrated (Figure 4). The rate of exchange at 37° C, as determined by dextran gel filtration, is shown in Figure 5.

Absorption tests. For these experiments, the binding sites of human and rat gastric juice were saturated with radioB_{12} as described above. $Co^{60}B_{12}$ bound to human gastric juice was used for Schilling tests in patients with pernicious anemia; fecal excretion tests in gastrectomized rats were performed with $Co^{57}B_{12}$ bound to rat gastric juice.

The results of a series of Schilling tests carried out in six patients with pernicious anemia are summarized in Table II. A separate Schilling test was performed after the oral administration of each of the following: free $Co^{57}B_{12}$, bound $Co^{60}B_{12}$, a mixture of the two prepared immediately before administration, and a mixture of free and bound radioB_{12} incubated at pH 6.8 to 7.0 at 37° C for 18 hours. In each patient, the percentage of initially free $Co^{57}B_{12}$ excreted in the

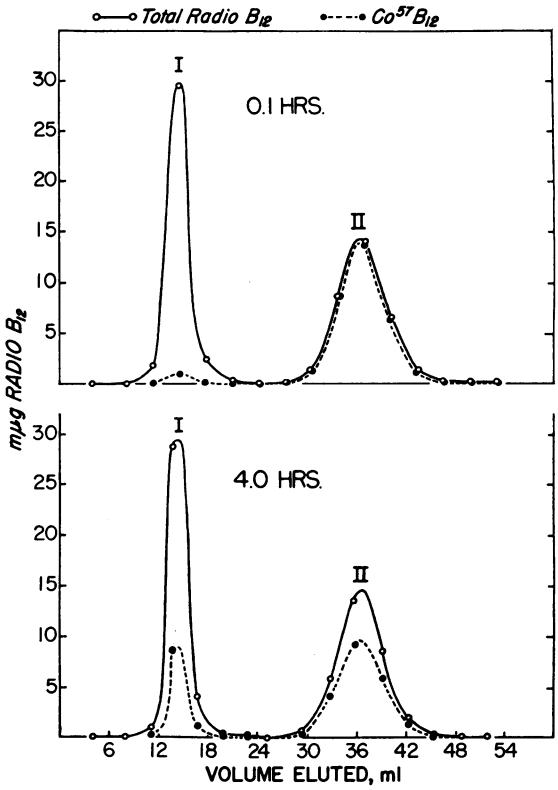

FIG. 4. DEMONSTRATION OF EXCHANGE BETWEEN FREE AND GASTRIC JUICE-BOUND VITAMIN B_{12} BY DEXTRAN GEL FILTRATION. A mixture of equal quantities of free $Co^{57}B_{12}$ and human gastric juice-bound $Co^{60}B_{12}$ was incubated at 37° C. At intervals, 2-ml samples of the mixture were applied to dextran gel columns. The elution pattern is shown for samples incubated for 0.1 and 4.0 hours. The solid lines represent total radioB_{12} ($Co^{57}B_{12}$ plus $Co^{60}B_{12}$), whereas the dashed lines represent $Co^{57}B_{12}$ alone. With incubation, an increasing amount of $Co^{57}B_{12}$ was found in peak I ("bound peak") while an equal amount of $Co^{57}B_{12}$ disappeared from peak II ("free peak"). The total radioB_{12} in each peak remained constant.

TABLE II

Absorption of free and bound radioactive cyanobalamin (radioB_{12}) by patients with pernicious anemia

Patient†	Radioactivity excreted in urine					
	Experiment I*		Experiment II*		Experiment III*	
	Co^{57} B_{12} (free)	Co^{60} B_{12}† (bound)	Co^{57} B_{12} (free)	Co^{60} B_{12}† (bound)	Co^{57} B_{12} (free)	Co^{60} B_{12}† (bound)
	%	%	%	%	%	%
1	1.4	13.7	5.0	14.4	5.8	8.8
2	1.7	16.8	5.0	12.2	8.0	10.5
3	2.7	17.2	4.2	10.6	5.7	6.3
4	2.3	19.8	4.8	14.3	7.1	10.0
5	5.6	23.4	10.0	16.3	11.2	12.6
6	1.0	14.9	4.2	8.6	5.0	6.2
Mean	2.4	17.6	5.5	12.7	7.2	9.1

* Experiment I: Co^{57} B_{12} and Co^{60} B_{12} given separately at least 1 week apart. Experiment II: Co^{57} B_{12} and Co^{60} B_{12} given together without prior incubation. Experiment III: Co^{57} B_{12} and Co^{60} B_{12} given together after prior incubation of the mixture at 37° C for 18 hours.

† Each patient received the same gastric juice specimens in all 3 experiments.

urine was observed to be higher when the unincubated mixture was given than when free $Co^{57}B_{12}$ was given alone. In five of the six subjects, there was concomitant decrease in excretion of initially bound $Co^{60}B_{12}$. These changes were even more marked when the mixture was incubated before its administration.

Similar experiments were carried out in two totally gastrectomized rats, except that in this case $Co^{57}B_{12}$ was bound to rat gastric juice and $Co^{60}B_{12}$ was given as the free vitamin. As shown in Figure 6, the results of these experiments paralleled those obtained in patients with pernicious anemia. Absorption of initially free $Co^{60}B_{12}$ was enhanced in the presence of gastric juice fully saturated with

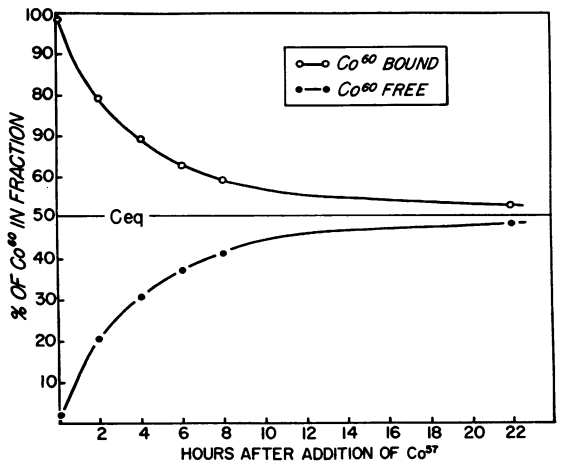

FIG. 5. KINETICS OF EXCHANGE BETWEEN FREE AND BOUND RADIOB_{12} AS DETERMINED BY DEXTRAN GEL FILTRATION. The experimental conditions were the same as described in Figure 4. If complete exchange occurred, then 50% of both the free and bound fractions would consist of $Co^{60}B_{12}$. The plot above illustrates the approach to this equilibrium (Ceq) for $Co^{60}B_{12}$ only. The plot for $Co^{57}B_{12}$ is superimposable, thus demonstrating true exchange.

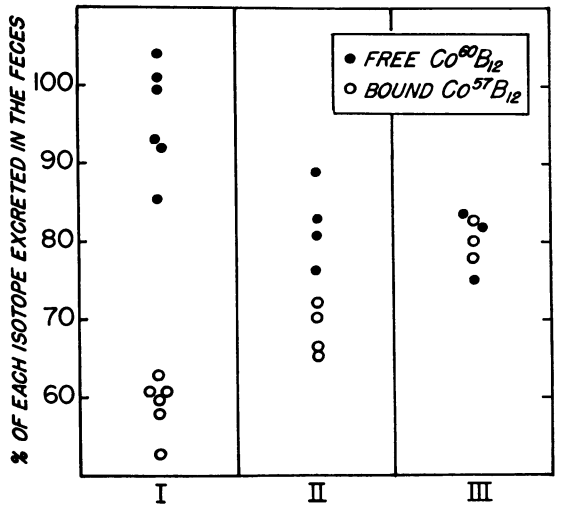

FIG. 6. ABSORPTION OF FREE AND GASTRIC JUICE-BOUND RADIOB_{12} BY TOTALLY GASTRECTOMIZED RATS. In 2 rats, fecal excretion of radioactivity was measured for 7 days after oral administration of 10 mμg of free $Co^{60}B_{12}$, or 10 mμg of $Co^{57}B_{12}$ that had been bound to rat gastric juice, or both. In experiment I, free $Co^{60}B_{12}$ and bound $Co^{57}B_{12}$ were administered at least 1 week apart. In experiment II, free and bound radioB_{12} were given immediately after mixing the two. In experiment III, the mixture of free $Co^{60}B_{12}$ and bound $Co^{57}B_{12}$ was incubated for 24 hours at 37° C before its administration.

TABLE III

Total vitamin B_{12} in rat tissue extracts as determined by Euglena gracilis *assay*

Tissue	Vitamin B_{12}*	Amount dialyzable
		%
Intestinal juice	11.7†	73
Everted intestinal sac	1.4‡	42
Washed intestinal homogenate	3.9	48
Washed liver homogenate	7.4	25

* Expressed as millimicrograms per total intestinal juice from a single rat or per 100 mg of tissue.
† Average of 3 determinations.
‡ Average of 4 experiments.

$Co^{57}B_{12}$. At the same time, absorption of initially bound $Co^{57}B_{12}$ was correspondingly diminished in the presence of free $Co^{60}B_{12}$. Absorption of $Co^{57}B_{12}$ and $Co^{60}B_{12}$, as determined by fecal excretion measurements, was approximately equal when the mixture was incubated before its administration. Absorption of total radioB_{12} ($Co^{57}B_{12}$ plus $Co^{60}B_{12}$) was the same whether the free and bound radioB_{12} were given separately or in either of the mixtures.

Exchange between gastric juice-bound radioB_{12} and vitamin B_{12} obtained from tissues of the rat. Since exchange in the above experiments had been determined by use of two radioisotopic species of cyanocobalamin, it was of some interest to determine whether naturally occurring vitamin B_{12} could also participate in such a process. This might have considerable bearing on the interpretation of various *in vitro* experiments employed by other workers to assess the absorption-promoting properties of IF. The techniques used to determine the extent to which vitamin B_{12} escaped from tissues (see Methods) were therefore designed to simulate the conditions under which most of these *in vitro* studies have been performed.

Table III lists the amounts of total and dialyzable vitamin B_{12} found in these studies. A single, gentle flushing of the intestinal lumen with a small quantity of KRB yielded considerable amounts of vitamin B_{12} as determined by *Euglena* assay. This vitamin B_{12} was considered to have been present in the intestinal juice, and 73% of it was found to be dialyzable. When everted sacs of intestine were washed and incubated with KRB for 1 hour, additional vitamin B_{12} activity was found in the medium bathing the mucosal surface and 42% was dialyzable. About twice as much activity was leached out of intestinal segments that were homogenized and washed before incubation, but the proportion of dialyzable material remained the same. Washed liver homogenates treated in a similar fashion yielded a still larger amount of vitamin B_{12}, but only 25% of this was dialyzable.

In order to determine whether the substance that was biologically active in the *Euglena* assay was capable of exchange with cyanocobalamin bound to gastric juice, the following experiment was performed. Two ml of rat gastric juice with binding sites completely saturated with 9.1 mμg of $Co^{57}B_{12}$ was placed in each of five dialysis bags, which were then individually dialyzed for 24 hours at 37° C at pH 7.4 against 5 ml of each of the tissue extracts. In a control experiment, one bag was dialyzed against 5 ml of KRB. After this initial dialysis, the bags were removed and dialyzed against a large volume of 0.15 M NaCl at 6° C for 48 hours in order to remove unbound material.

The first two columns of Table IV list the total and dialyzable vitamin B_{12} present in the various

TABLE IV

Exchange of gastric juice-bound $Co^{57}B_{12}$ with vitamin B_{12} extracted from tissues

Tissue extract	Vitamin B_{12}*		Final bag contents (after second dialysis)		Theoretical Co^{57} B_{12}‡
	Total	Dialyzable	Vitamin B_{12}†	Co^{57} B_{12}	
	mμg	mμg	mμg	mμg	mμg
Intestinal juice	12.5	9.1	10.0	3.4	4.5
Everted intestinal sac extract	1.9	0.8	8.3	8.1	8.4
Homogenized intestinal extract	4.2	2.0	8.6	7.9	7.5
Homogenized liver extract	11.2	2.8	9.4	6.9	6.9
Krebs-Ringer-bicarbonate solution (control)	0	0	8.8	8.7	9.1

* Amount present by *Euglena* assay in fluids against which Co^{57} B_{12} bound to gastric juice was initially dialyzed.
† By *Euglena* assay.
‡ Assuming complete exchange during initial dialysis.

tissue extracts against which the gastric juice-bound $Co^{57}B_{12}$ was initially dialyzed. The third and fourth columns record, respectively, the total vitamin B_{12} content as determined by bioassay and the $Co^{57}B_{12}$ remaining in the dialysis bag at the completion of the second dialysis. The last column shows the amounts of $Co^{57}B_{12}$ theoretically present in the dialysis bags at the end of the second dialysis if complete exchange during the first dialysis is assumed.

In general, the greater the amount of dialyzable vitamin B_{12} in the extracts, the lower the activity of $Co^{57}B_{12}$ remaining in the bags at the completion of the experiment, and considering the experimental errors involved, the expected and observed values agree well. Also, the total content of vitamin B_{12} remaining in the bags after the second dialysis was approximately equal to that present at the beginning of the experiment (9.1 mμg), indicating that the amount of $Co^{57}B_{12}$ displaced from the gastric juice was replaced with an equivalent amount of tissue-extracted vitamin. In three other experiments, the protocol was similar, except that the total vitamin B_{12} content remaining in the bags was not measured. In all experiments, the reduction in final bag radioactivity was proportional to the vitamin B_{12} content of the tissue extracts. Thus, in whatever chemical form vitamin B_{12} may exist in intestinal juice or in tissue extracts, its biological activity in the *Euglena* assay appeared to be proportional to its ability to exchange with gastric juice-bound radioB_{12}.

TABLE V

*Rate of exchange between free radioB_{12} and radioB_{12} bound to human gastric juice as determined by different methods for measuring binding**

Incubation	Percentage of theoretically complete exchange		
	Dialysis	Gel filtration	Starch gel electrophoresis
hours			
2	39 [36–43]	43 [41–45]	
4	56 [52–61]	61 [59–64]	70 [67–73]
8	75 [66–80]	84 [81–88]	
24	84 [79–87]	96 [95–97]	95 [93–97]
No. of experiments	7	3	2

* In all experiments, free and bound radioB_{12} were mixed in approximately equal quantities and incubated at 37° C. Average values are given, with ranges in brackets.

DISCUSSION

The meaning of the phrase "binding of vitamin B_{12}" depends upon the methods used to measure this binding (11). In the present study, a significant exchange between free and gastric juice-bound radioB_{12} could be demonstrated *in vitro* whether binding was determined by dialysis, by electrophoresis, or by dextran gel filtration. At 37° C, exchange between free and bound radioB_{12} was approximately 40% complete within 2 hours (Table V). At lower temperatures, a much slower rate of exchange was observed. Highley and Ellenbogen (20) have recently shown that only 10% of hog IF-bound radioB_{12} was made dialyzable by incubation with an equal quantity of unlabeled cyanocobalamin at 4° C for 24 hours, i.e., only 20% of theoretically complete exchange had occurred. These results agree with our observations at 6° C.

IF cannot be equated with the binding activity of gastric juice, since vitamin B_{12}-binding substances that lack IF activity are present in gastric juice (21). The exchange observed between free and gastric juice-bound radioB_{12} may have involved only nonspecific binding substances unrelated to IF. Absorption studies, however, in patients and rats lacking IF showed that: *1*) the absorption of free radioB_{12} was consistently enhanced when it was administered with gastric juice fully saturated with an equal amount of radioB_{12}; *2*) at the same time, bound radioB_{12} was absorbed more poorly when it was given with free radioB_{12} than when it was given alone; and *3*) when a mixture of equal amounts of free and bound radioB_{12} was incubated at 37° C for long periods before it was administered, the free and bound radioB_{12} were absorbed to approximately the same extent. These results indicate that IF may share in the exchange observed *in vitro* between free and gastric juice-bound radioB_{12}.

The correlations observed in the present study between gastric juice binding and absorption of cyanocobalamin are consistent with the concept (3) that binding of vitamin B_{12} may play an important role in the function of IF. The results obtained confirm previous observations in man (22, 23) which suggest that, in the presence of free cyanocobalamin, gastric juice-bound cyanocobalamin is preferentially absorbed.

In contrast to our findings, Toporek (22) concluded from similar experiments that exchange

between free and gastric juice-bound cyanocobalamin was "slow or negligible." The data of Schilling and Schloesser (23) tend to support Toporek's conclusion. This discrepancy may be due to differences in experimental techniques, since in both studies only small changes in the percentage of excretion of radioactivity were possible with the large doses of cyanocobalamin used, and the results of giving gastric juice-bound radioB_{12} alone were not reported. Even so, the results in six of eight of Toporek's patients could be interpreted as demonstrating some degree of exchange.

It was demonstrated by *Euglena gracilis* assay that vitamin B_{12} was present in the intestinal juice of rats in quantities approaching the amounts of radioB_{12} given in various absorption studies (24–26). The assay method is not specific and will measure pseudovitamin B_{12} as well as cyanocobalamin and cobamide coenzymes (27–29). Since the material found in intestinal contents was able to exchange with gastric juice-bound radioB_{12}, it is unlikely that this material consisted only of pseudovitamin B_{12}, which does not compete effectively with radioB_{12} for gastric juice-binding sites (30).

It should be emphasized that cyanocobalamin does not exist as such in nature and that the vitamin probably occurs naturally as a cobamide coenzyme (31). In whatever form the unlabeled vitamin B_{12} exists in intestinal juice, it appears to be able to displace radioB_{12} from the binding sites of gastric juice and may therefore effectively compete with gastric juice-bound radioB_{12} for absorption sites.

The presence of unlabeled vitamin B_{12} in intestinal contents can be attributed to the significant quantities of the vitamin that have been found in bile (8, 9). Intestinal excretion of unlabeled vitamin might also occur, since it has been observed both in the present study and previously (32) that vitamin B_{12} is released *in vitro* from the mucosal surface of everted intestinal sacs.

It would appear that absorption tests that measure radioactivity after the oral administration of radioB_{12} cannot be interpreted as representing an absolute estimation of total vitamin B_{12} absorption. As a result of exchange with unlabeled vitamin B_{12} present in the intestinal lumen, the amount of radioB_{12} that is bound to IF would tend to diminish during its passage down the small bowel. Total absorption of the vitamin would therefore be underestimated if calculated only on the basis of the specific activity of the administered radioB_{12}. The difference between actual and apparent absorption would vary depending upon: *1*) the relative amount of unlabeled vitamin available for exchange, *2*) the length of time during which exchange could take place in the intestine, and *3*) intraluminal factors, such as the presence of vitamin B_{12}-binding substances, which might affect the rate of exchange.

Unlabeled vitamin B_{12} present in the intestine could also become bound by any available IF and could then compete with IF-bound radioB_{12} for absorption sites. Again total absorption of the vitamin would be greater than apparent absorption based on radioactivity measurements. It seems unlikely that unlabeled vitamin could interfere with absorption of IF-bound radioB_{12} on the basis of simple isotope dilution, since it has been shown that the IF-dependent and IF-independent mechanisms for vitamin B_{12} absorption are entirely different (33). Therefore, in order to interfere with the physiologic mechanism of radioB_{12} absorption, unlabeled vitamin must either become bound to available IF or exchange with radioB_{12} that is already bound.

Decreased absorption of radioB_{12} is observed when a relatively large amount of cyanocobalamin is given parenterally before the oral administration of radioB_{12} (34, 35). Although this phenomenon might be due to "saturation" of intramural intestinal receptors (36), it is possible that some of the parenterally administered cyanocobalamin is excreted into the intestine, where it could either exchange with bound radioB_{12} or be bound by any available IF. Similarly, the inhibitory effect of rat bile on radioB_{12} absorption (37) has been attributed to unlabeled vitamin B_{12} present in the bile (9). This unlabeled vitamin B_{12} might interfere with radioB_{12} by the mechanisms discussed above. We would conclude that the interpretation of absorption tests which utilize radioB_{12} must take into account the possibility of exchange between gastric juice-bound radioB_{12} and unlabeled vitamin B_{12} present in the intestinal lumen.

Studies of tissue uptake of radioB_{12} *in vitro* have provided evidence concerned with the mechanism of vitamin B_{12} absorption (6) and have led to methods for the assay of human IF (38, 39).

Systems in which uptake of radioB_{12} is measured, however, may be more complex than has previously been supposed. In the present investigation, endogenous vitamin B_{12} was observed to escape from rat liver and intestine into the incubating medium in quantities exceeding the amounts of radioB_{12} previously reported to be taken up by these tissues. Turner and Hughes (32) have described quantitatively similar losses of endogenous vitamin B_{12} from the mucosal surface of everted intestinal sacs. Thus simple measurement of radioactivity may not necessarily indicate that net uptake of vitamin B_{12} by a tissue has actually taken place. When the effects of gastric juice on tissue uptake of radioB_{12} are studied *in vitro,* interpretation of results is further complicated by the ability of tissue vitamin B_{12} that leaks into the incubation medium to exchange with radioB_{12} bound to gastric juice.

The mechanism by which tissue uptake of radioB_{12} is altered by factors such as temperature (40, 41), pH (42), calcium or EDTA (42, 43), or IF itself may in part depend upon the amount of endogenous vitamin that escapes from the tissue. The uncontrolled presence of endogenous vitamin B_{12} could unpredictably distort the quantitative effects of various substances, including IF, on the tissue uptake of radioB_{12}. The impact of inhibitory factors might be exaggerated, whereas the effects of factors which stimulate radioB_{12} uptake might be minimized. It would appear that *in vitro* studies of tissue uptake of radioB_{12} should include data concerning the quantities of unlabeled vitamin B_{12} present in the system being studied.

Finally, it has been suggested that radioB_{12} is "released" from IF by a "releasing factor" present in intestinal extracts (44–46), or in extracts of other tissues (47). In the present study, "release" of radioB_{12} from gastric juice binders occurred when bound radioB_{12} was placed inside a dialysis bag and intestinal juice, or extracts of liver, or intestinal homogenates were placed outside the bag. When the total vitamin B_{12}-like material present was measured, it appeared that this "release" of radioB_{12} could be best explained by exchange between the bound radioB_{12} and the unlabeled vitamin B_{12} derived from tissues. Such an exchange does not explain all of the experiments that have suggested the presence of a "releasing factor" in the intestine. For example, mere exchange cannot account for the species-specific action of "releasing factor" which has been reported in rat (44, 48) and human (48) intestine. Nevertheless, it would appear that in studies concerned with the "release" of radioB_{12} from binding substances, a distinction should be made between exchange and true release.

SUMMARY

Exchange between free and gastric juice-bound radioactive cyanocobalamin was demonstrated *in vitro* when free and bound cyanocobalamin were separated by dialysis, by starch gel electrophoresis, or by dextran gel filtration. Absorption studies in patients with pernicious anemia and in gastrectomized rats suggested that intrinsic factor was involved in this exchange.

Significant quantities of endogenous vitamin B_{12} were found in the intestinal lumen of rats. When rat liver or intestine was incubated, tissue vitamin B_{12} escaped into the incubation medium. Exchange between this endogenous vitamin B_{12} and gastric juice-bound radioactive cyanocobalamin was demonstrated.

These findings are pertinent to the quantitative interpretation of studies concerned with *in vitro* tissue uptake and *in vivo* absorption of radioactive cyanocobalamin.

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REFERENCES

1. Donaldson, R. M., Jr., J. H. Katz, and J. D. DiMase. Demonstration of an exchange between free and intrinsic factor-bound vitamin B_{12} (abstract). J. clin. Invest. 1962, **41**, 1354.
2. Gräsbeck, R. Physiology and pathology of vitamin B_{12} absorption, distribution, and excretion. Advanc. clin. Chem. 1960, **3**, 299.
3. Wijmenga, H. G. Intrinsic factor and vitamin B_{12}-binding substances *in* Vitamin B_{12} and Intrinsic Factor, H. C. Heinrich, Ed. Stuttgart, Ferdinand Enke, 1957, p. 156.

4. Bird, O. D., and B. Hoevet. The vitamin B_{12}-binding power of proteins. J. biol. Chem. 1951, **190**, 181.
5. Gregory, M. E. Microbiological assay of 'vitamin B_{12}' in the milk of different animal species. Brit. J. Nutr. 1954, **8**, 340.
6. Herbert, V. Studies on the role of intrinsic factor in vitamin B_{12} absorption, transport, and storage. Amer. J. clin. Nutr. 1959, **7**, 433.
7. Abels, J., J. J. M. Vegter, M. G. Woldring, J. H. Jans, and H. O. Nieweg. The physiologic mechanism of vitamin B_{12} absorption. Acta med. scand. 1959, **165**, 00.
8. Reizenstein, P. G. Excretion, enterohepatic circulation, and retention of radiovitamin B_{12} in pernicious anemia and in controls. Proc. Soc. exp. Biol. (N. Y.) 1959, **101**, 703.
9. Herbert, V., and M. E. Kaplan. Studies suggesting the presence of intrinsic factor in bile. Proc. Soc. Biol. (N. Y.) 1961, **107**, 900.
10. Kay, A. W. Effect of large doses of histamine on gastric secretion of HCl; an augmented histamine test. Brit. med. J. 1953, **2**, 77.
11. Gräsbeck, R. Studies on the vitamin B_{12}-binding principle and other biocolloids of human gastric juice. Acta. med. scand. 1956, **154**, suppl. 314.
12. Schilling, R. F. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. J. Lab. clin. Med. 1953, **42**, 860.
13. Strauss, E. W., R. M. Donaldson, Jr., and F. H. Gardner. A relationship between intestinal bacteria and the absorption of vitamin B_{12} in rats with diverticula of the small bowel. Lancet 1961, **2**, 736.
14. Smithies, O. An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals. Biochem. J. 1959, **71**, 585.
15. Jeffries, G. H., F. W. Smith, D. W. Hoskins, and M. H. Sleisenger. Fractionation of the non-dialyzable components of human gastric juice by starch gel electrophoresis. Gastroenterology 1961, **41**, 467.
16. Kakei, M., and G. B. J. Glass. Separation of bound and free vitamin B_{12} on sephadex column (abstract). Fed. Proc. 1962, **21**, 469.
17. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. Manometric Techniques, revised ed. Minneapolis, Burgess, 1957, p. 149.
18. Wilson, T. H., and G. Wiseman. The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. (Lond.) 1954, **123**, 116.
19. Ross, G. I. M. Vitamin B_{12} assay in body fluids using *Euglena gracilis*. J. clin. Path. 1952, **5**, 250.
20. Highley, D. R., and L. Ellenbogen. Studies on the mechanism of vitamin B_{12} absorption: *in vivo* transfer of vitamin B_{12} to intrinsic factor. Arch. Biochem. 1962, **99**, 126.
21. Glass, G. B. J., and H. Uchino. Vitamin B_{12} binders in human gastric juice. Fed. Proc. 1959, **18**, 53.
22. Toporek, M. The relation of binding power to intrinsic factor activity. Effect of pseudovitamin B_{12} on absorption of vitamin B_{12}. Amer. J. clin. Nutr. 1960, **8**, 297.
23. Schilling, R. F., and L. L. Schloesser. Intrinsic factor studies. V. Some aspects of the quantitative relationship between vitamin B_{12}, intrinsic factor, binding, and the absorption of vitamin B_{12} *in* Vitamin B_{12} and Intrinsic Factor, H. S. Heinrich, Ed. Stuttgart, Ferdinand Enke, 1957, p. 194.
24. Watson, G. M., and H. W. Florey. The absorption of vitamin B_{12} in gastrectomized rats. Brit. J. exp. Path. 1955, **36**, 479.
25. Nieweg, H. O., A. Arends, E. Mandema, and W. B. Castle. Enhanced absorption of vitamin B_{12} in the gastrectomized rat by rat intrinsic factor. Proc. Soc. exp. Biol. (N. Y.) 1956, **91**, 328.
26. Moertel, C. G., H. H. Scudamore, C. A. Owen, Jr., and J. L. Bollman. Intestinal absorption of cobalt^{60}-labeled vitamin B_{12} in the normal male albino rat. Amer. J. Physiol. 1959, **197**, 347.
27. Kon, S. K. Other factors related to vitamin B_{12} *in* The Biochemistry of Vitamin B_{12}, Biochemical Society Symposia No. 13, R. T. Williams, Ed. Cambridge, Cambridge University Press, 1955, p. 17.
28. Ford, J. E., and S. H. Hutner. Role of vitamin B_{12} in the metabolism of microorganisms. Vitam. and Horm. 1955, **13**, 101.
29. Beck, W. S. The metabolic functions of vitamin B_{12}. New Engl. J. Med. 1962, **26**, 708.
30. Bunge, M. B., L. L. Schloesser, and R. F. Schilling. Intrinsic factor studies. IV. Selective absorption and binding of cyanocobalamin by gastric juice in the presence of excess pseudovitamin B_{12} or 5,6-dimethylbenzimidazole. J. Lab. clin. Med. 1956, **48**, 735.
31. Toohey, J. L., and H. A. Barker. Isolation of coenzyme B_{12} from liver. J. biol. Chem. 1961, **236**, 560.
32. Turner, J. B., and D. E. Hughes. The absorption of some B-group vitamins by surviving rat intestine preparations. Quart. J. exp. Physiol. 1962, **47**, 107.
33. Doscherholmen, A., and P. S. Hagen. A dual mechanism of vitamin B_{12} plasma absorption. J. clin. Invest. **36**, 1551.
34. Baker, S. J., and D. L. Mollin. The relationship between intrinsic factor and the intestinal absorption of vitamin B_{12}. Brit. J. Haemat. 1955, 1, 46.
35. Callender, S. T., and J. R. Evans. The urinary excretion of labelled vitamin B_{12}. Clin. Sci. 1955, **14**, 295.
36. Glass, G. B. J., L. J. Boyd, and L. Stephanson. Intestinal absorption of vitamin B_{12} in man. Science 1954, **120**, 74.

37. Johnson, P. C., T. B. Driscoll, and W. L. Honska. Inhibition of vitamin B_{12} absorption by bile. Proc. Soc. exp. Biol. (N. Y.) 1961, **106**, 181.
38. Sullivan, L. W., V. Herbert, and W. B. Castle. *In vitro* assay for human intrinsic factor (abstract). Clin. Res. 1962, **10**, 208.
39. Castro, Z., and G. B. J. Glass. Guinea pig intestinal homogenate system (GPIMH) for assay of I. F. activity (abstract). Clin. Res. 1962, **10**, 197.
40. Herbert, V. Development of a possible *in vitro* assay for intrinsic factor. Proc. Soc. exp. Biol. (N. Y.) 1958, **97**, 668.
41. Herbert, V. Mechanism of intrinsic factor action in everted sacs of rat small intestine. J. clin. Invest. 1959, **38**, 102.
42. Herbert, V., and W. B. Castle. Divalent cation and pH dependence of rat intrinsic factor action in everted sacs and mucosal homogenates of rat small intestine. J. clin. Invest. 1961, **40**, 1978.
43. Cooper, B. A., W. Paranchych, and L. Lowenstein. Studies on the absorption by guinea pig intestine of cyanocobalamin incubated with intrinsic factor. J. clin. Invest. 1962, **41**, 370.
44. Cooper, B. A., and W. B. Castle. Sequential mechanisms in the enhanced absorption of vitamin B_{12} by intrinsic factor in the rat. J. clin. Invest. 1960, **39**, 199.
45. Herbert, V., B. A. Cooper, and W. B. Castle. The site of vitamin B_{12}-intrinsic factor complex "releasing factor" activity in the rat small intestine. Proc. Soc. exp. Biol. (N. Y.) 1962, **110**, 315.
46. Doscherholmen, A. Enzymatic release of vitamin B_{12} bound either to the intrinsic factor or in the wall of the small intestine. Int. Z. Vitamin-forsch. 1961, **31**, 380.
47. Nyberg, W., M. Saarnw, and R. Grasbeck. The releasing of vitamin B_{12} from complex-bound form by a principle contained in various human organs. Int. Z. Vitamin-forsch. 1961, **31**, 380.
48. Herbert, V. Mechanisms of absorption of vitamin B_{12}. Fed. Proc. 1960, **19**, 884.