

SEQUENTIAL MECHANISMS IN THE ENHANCED ABSORPTION OF VITAMIN B₁₂ BY INTRINSIC FACTOR IN THE RAT *

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Pernicious anemia, a clinical state of vitamin B₁₂ deficiency, is characterized by a virtually complete absence of gastric intrinsic factor secretion, resulting in marked inability of the patient to absorb the low concentrations of vitamin B₁₂ present in usual dietary sources. Intrinsic factor deficiency is also produced by total gastrectomy in the rat and, as in the patient, can be temporarily abolished by the oral administration of intrinsic factor. A number of facts concerning the nature and function of intrinsic factor have been discovered by observations on patients (1-2) and, recently, on gastrectomized rats (3-5).

Intrinsic factor preparations of various species—man, hog, rat and dog—are capable of binding vitamin B₁₂ (6-9) as are other substances such as food proteins which, however, lack intrinsic factor activity (10, 11). Vitamin B₁₂ bound by intrinsic factor is preferentially absorbed over unbound vitamin when both are simultaneously administered in pernicious anemia (12) as well as in the intestine of the gastrectomized rat (13). These facts suggest that normally the first task of the gastric intrinsic factor may be to compete for the vitamin B₁₂ of ingested animal protein with which the vitamin is associated or bound. For convenience in reporting the present experiments, we shall refer to this competition as Phase I of the hypothetical action of intrinsic factor. That vitamin B₁₂ bound to intrinsic factor is preferentially absorbed also suggests that the vitamin must approach, adhere to, and perhaps even penetrate the intestinal wall in combination with intrinsic factor. It has been stated that binding of vitamin B₁₂ to rat intestinal segments *in vitro* is greatly enhanced by both hog (14) and rat (15) intrinsic factor-containing

preparations. This process apparently requires the presence of calcium ions (14). However, because hog intrinsic factor does not enhance absorption of vitamin B₁₂ by the gastrectomized rat (3-5), the physiological significance of experiments with hog stomach preparations requires evaluation. Thus adherence of vitamin B₁₂ to the intestinal wall after binding to intrinsic factor may be only a preparatory step (Phase II) for subsequent species-related transport across the intestinal wall. At any rate, there is evidence from observations in both man (16, 17) and in the rat (18) that a process concerned with the absorption of vitamin B₁₂ takes place in the intestinal wall and requires up to four hours. Species-related activity would be expected to be characteristic of a process occurring within a tissue but not necessarily upon its surface, in this case the small intestine. Consequently, as in a recent preliminary report of this work (19), we shall refer for convenience to the transport of the vitamin across the intestine and into the blood stream as Phase III of the action of intrinsic factor.

MATERIALS AND GENERAL METHODS

Various sources of intrinsic factor. Rat gastric juice was collected by the method of Shea (20) using ether anesthesia. Five hours of such pyloric ligation was found to give a 5 to 10 ml. yield from the distended stomach of a fasted but well hydrated rat. Using 20 per cent sodium hydroxide solution, the juice was immediately neutralized to pH 7.0, as determined on a Beckman pH meter and kept frozen in aliquots at -20° C. until used.

Rat stomach extract was prepared from freshly frozen rat stomachs stored at -20° C. The stomachs were thawed, homogenized in water in a Waring blender, centrifuged, and the sediment discarded. One part of concentrated hydrochloric acid solution was added to each nine parts of the extract, and the precipitate was removed by centrifugation. The extract was then neutralized to pH 7.0 with 20 per cent sodium hydroxide (w/v), dialyzed against water at 4° C. for 18 hours, and thereafter concentrated at room temperature in a cellophane casing suspended before an electric fan to a volume correspond-

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ing to 2 ml. of extract per stomach. One-half ml. of this material was found to be active in reducing the 100 per cent fecal excretion of radioactivity of gastrectomized rats fed 0.015 μ g. of radioactive vitamin B_{12} to 70 per cent of the administered dose. Normal rats excrete about 50 per cent of this dose of $Co^{60}-B_{12}$.

Human gastric juice was collected by Histalog®¹ stimulation of normal donors after gastric intubation. The juice was filtered through cheesecloth, allowed to stand at pH 10 for 20 minutes in order to destroy pepsin (21) and then neutralized to pH 7. The gastric juice from five donors was pooled and stored frozen at -20° C. in small aliquots.

Hog intrinsic factor preparation No. Wes 671-A is a whitish powder,² reported to have no inhibitory action in pernicious anemia. This preparation was suspended in a concentration of 0.1 per cent (w/v) in water kept at 4° C. and diluted 1:20 in water just before use. Four mg., when given with 15 μ g. of vitamin B_{12} is stated to represent 1 U.S.P. unit (oral) of Vitamin B_{12} with Intrinsic Factor Concentrate.

Other experimental reagents. Rat intestinal extract was prepared by homogenizing the entire small intestine of a 250 Gm. male rat in 50 ml. of distilled water in a Waring blender. This homogenate was then centrifuged in a laboratory angle centrifuge, and the slightly opalescent supernatant was frozen at -20° C. in small aliquots.

Krebs-Ringer phosphate at pH 7.4 was prepared fresh daily, from stock solutions, as described by Umbreit, Burris and Stauffer (22).

Other assay methods employed. Radioactive vitamin B_{12} , $Co^{60}-B_{12}$, with a specific activity of approximately 1 μ c. per μ g.³ was used in a concentration of 0.03 μ g. per ml. of water. Partition coefficients between benzyl alcohol and phosphate buffer at pH 7.0 ranged from 0.75 to 0.80, indicating that the solution contained between 90 and 100 per cent cyanocobalamin (23). Radioactive counting of various solutions containing $Co^{60}-B_{12}$ was carried out in a well-type scintillation counter.

Nonradioactive vitamin B_{12} was determined microbiologically using *Euglena gracilis* var. *bacillaris* according to the method of Ross (24).

INVESTIGATIONS

The present experiments represent attempts to add further information concerning the characteristics of each of the three hypothetical phases of intrinsic factor action mentioned in the introduction.

¹ Parenteral preparation of 3-beta-aminoethylpyrazole dihydrochloride (Lilly).

² Obtained through the kindness of Dr. Thomas Jukes, American Cyanamid Company, Pearl River, N. Y.

³ Obtained from Merck and Company, Rahway, N. J.

Phase I: Competition of intrinsic factor for vitamin B_{12} of ingested food proteins

In these experiments the rates of diffusion through cellophane membranes of vitamin B_{12} from homogenized suspensions of lean beef and pork muscle and from a solution of casein were compared with the diffusion rate of vitamin B_{12} from normal human gastric juice at various pH values.

Experiments with added $Co^{60}-B_{12}$. One per cent Hammarsten casein solution, 1 per cent lean beef muscle, and the centrifuged supernatant as well as the sediment of a 1 per cent lean pork muscle suspension, respectively, were incubated with 0.006 μ g. of $Co^{60}-B_{12}$ at room temperature in the dark for one hour. Equal volumes of these solutions were then placed in screw-capped test tubes, and 0.5 ml. of neutralized human gastric juice was added to selected tubes. An equal volume of distilled water was added to the remainder of the tubes. Visking casing, 8 mm. in diameter and 15 cm. in length with the ends tightly tied in order to form a bag, was used as a semipermeable membrane. In order to equalize osmotic pressure differences, equal volumes of appropriate aliquots of the casein, pork or beef muscle suspensions, but without added $Co^{60}-B_{12}$, were placed inside the visking bags. The bags were folded over, with an air bubble in each limb, and inserted into the test tubes, which were then closed with "parafilm" and a screw cap. Dialysis was then carried out while the test tubes, which contained an air space, were rotated end over end at the rate of 6 rpm in an incubator in the dark at 37° C. for five hours.

Duplicate experiments were run at pH 3.5 and pH 7.0, by adjustment of the original suspension before division between the test tube and the interior of the cellophane bag with 2 N hydrochloric acid or with 0.1 N sodium hydroxide solution. In other experiments preliminary incubation of cellophane bags in 0.85 per cent saline at pH 3.5 for five hours demonstrated no detectable effect of this treatment on the membrane during subsequent dialyses at pH 3.5 or pH 7.0.

At the conclusion of each experiment the cellophane bag was removed from each test tube, quickly rinsed under running water and wiped dry. Two ml. of its contents, as well as of its

TABLE I

Radioactivity transferred during dialysis from "outside solution" (containing added Co⁶⁰-B₁₂) to an equal volume of "inside solution" (containing no added Co⁶⁰-B₁₂)

Composition of outside solution	pH	Composition of inside solution	Increase in radioactivity of inside solution
			%
Water	7.0	Water	47.5
Casein	7.0	Water	22.0
Casein	3.5	Water	32.0
Casein	7.0	Water + NHGJ*	25.0
Casein	3.5	Water + NHGJ	42.0
Casein + NHGJ	7.0	Casein	2.6
Casein + NHGJ	3.5	Casein	2.6
Pork muscle supernatant	7.0	Pork muscle supernatant	38.8
Pork muscle supernatant	3.5	Pork muscle supernatant	42.8
Pork muscle supernatant	7.0	Pork muscle supernatant + NHGJ	72.0
Pork muscle supernatant	3.5	Pork muscle supernatant + NHGJ	81.5
Pork muscle sediment	7.0	Pork muscle sediment	4.1
Pork muscle sediment	3.5	Pork muscle sediment	36.0
Pork muscle sediment	7.0	Pork muscle sediment + NHGJ	5.5
Pork muscle sediment	3.5	Pork muscle sediment + NHGJ	75.5
Beef muscle suspension	7.0	Beef muscle suspension	35.0
Beef muscle suspension	3.5	Beef muscle suspension	39.5
Beef muscle suspension	7.0	Beef muscle suspension + NHGJ	57.0
Beef muscle suspension	3.5	Beef muscle suspension + NHGJ	78.0

* Normal human gastric juice.

corresponding test tube, were then measured out using volumetric pipettes. The radioactivity of each solution was determined. The exchange of radioactivity from outer to inner solution was recorded as a percentage of the initial radioactivity of the outer solution. The sums of the radioactivity in both the inner and the outer solutions represented approximately all of the radioactivity originally introduced.

In certain experiments, dialysis was carried out in the dark without rotation at 37° C. for seven hours and at 4° C. for 18 hours. These procedures were found to give results identical to those of the procedure described above.

Results. As shown in Table I, added Co⁶⁰-B₁₂ diffused slowly at pH 7.0 from casein and pork muscle, and with less difficulty from beef muscle and from the soluble substances in pork muscle. This diffusibility was greater when dialysis took place at pH 3.5 and was also increased at both pH 3.5 and 7.0 by the presence of human gastric juice devoid of peptic activity on the opposite side of the cellophane membrane. On the other hand, when the same amount of Co⁶⁰-B₁₂ was added to human gastric juice there was essen-

tially no diffusion of the vitamin into casein at either pH 3.5 or pH 7.0.

Experiments with native vitamin B₁₂. As a check on the studies with added Co⁶⁰-B₁₂, similar dialysis experiments were carried out using suspensions of beef and pork muscle in which the native vitamin B₁₂ exchanged was determined microbiologically. Suspensions of 2 per cent beef and pork muscle were placed in test tubes and dialyzed against an equal volume of 12.5 per cent human gastric juice of known vitamin B₁₂ content at pH 6.0 and at pH 1.5. Twenty-five per cent normal human gastric juice in water was dialyzed against water at pH 6.0 and 1.5, respectively. After dialysis, the solutions inside the cellophane bags were heated to 100° C. in order to free the vitamin and were then analyzed microbiologically for their gain in vitamin B₁₂ content.

Results. As shown in Table II, dialysis of normal human gastric juice against beef and pork muscle suspensions at both pH 6.0 and 1.5 resulted in a marked increase in the native vitamin B₁₂ content of the gastric juice. This loss of vitamin B₁₂ to gastric juice appeared to be en-

TABLE II
Native vitamin B₁₂ transferred during dialysis from "outside solution" to an equal volume of "inside solution"

Composition of outside solution	pH	Composition	Inside solution	
			Vitamin B ₁₂	
			Before dialysis	After dialysis
			$\mu\text{g./ml.}$	$\mu\text{g./ml.}$
Pork muscle suspension, 25% in water	6.0	NHGJ*	104	1,304
	1.5	12.5% in water	104	1,384
Beef muscle suspension, 25% in water	6.0	NHGJ	104	1,904
	1.5	12.5% in water	104	7,144
Water	6.0	NHGJ	208	208
	1.5	25% in water	208	208

* Normal human gastric juice.

hanced at low pH values as in the experiment with added Co⁶⁰-B₁₂. On the other hand, dialysis of gastric juice against water at pH 1.5 and 6.0 resulted in no detectable loss of its native vitamin B₁₂ content at either pH.

Phase II: Enhanced adsorption by the intestinal wall of vitamin B₁₂ bound to intrinsic factor

In these experiments intraluminal perfusions were carried out through cannulae inserted into paired loops of rat ileum with intact blood supply. The effect of sources of intrinsic factor and of other substances upon the uptake of Co⁶⁰-B₁₂ by the intestine was determined by measurement of the differences in radioactivity of the paired intestinal segments removed at the end of each experiment.

The perfusion technique employed was that of Nieweg, Shen and Castle (13) except that it was conveniently discovered that intestinal segments of normal fasted rats, like those of gastrectomized rats, were sensitive to the influence of rat intrinsic factor. Consequently, intact male Sprague-Dawley rats weighing 150 to 250 Gm. were anesthetized with pentobarbital, 3.5 mg. per 100 Gm. of rat, given intraperitoneally. Two segments of small intestine, 10 cm. in length, were then isolated between fine plastic inflow and outflow catheters tied in place and carefully positioned to permit free passage of fluid from above downward through the lumen of each segment. By means of a pair of 1 ml. tuberculin syringes equipped with three-way stopcocks and rigidly supported by a burette stand, test and control

solutions could be simultaneously perfused at similar rates controlled by a stop watch through one or the other of the pair of intestinal segments. Although no consistent differences were detected, the upper and lower intestinal loops were alternately selected in successive animals for perfusion of the test and control solutions, respectively.

At the beginning of an experiment the intestinal loops were rinsed by perfusion with 2 ml. of warm Krebs-Ringer phosphate solution at the rate of 0.5 ml. per minute. The animal was then left undisturbed for three minutes, following which the perfusion of the test fluid was begun in one, while the control fluid was perfused at an identical rate through the other isolated loop. At first, 0.25 ml. of each perfusion fluid was injected every 15 seconds for one minute. This was then followed by 0.20 ml. of the same fluid at one minute intervals for 15 minutes. Thus, in all, 4.0 ml. of each solution was perfused in 17 minutes through each loop of intestine. Usually the perfusion fluid and its control were constituted to approximate Krebs-Ringer phosphate solution. Invariably each contained 0.015 $\mu\text{g.}$ of Co⁶⁰-B₁₂.

At the end of the perfusion the loops were "rinsed" by perfusion with 6 ml. of isotonic saline and then gently emptied of liquid by the introduction of 6 ml. of air through the inflow cannula. Each loop was then removed from the animal preparation, turned inside out on a metal rod, taken off, placed in a 25 ml. Erlenmeyer flask and shaken gently for five minutes in 4 ml. of the rinsing solution required by the particular experiment. The tissue was then removed,

drained of rinsing fluid, and dissolved in a test tube containing a weighed amount of concentrated sulfuric acid. The weight of each loop was determined by difference, and its radioactivity estimated in a scintillation counter.

Effects of rat and human gastric juice and of a hog intrinsic factor preparation on uptake of Co⁶⁰-B₁₂. In these experiments one intestinal loop was perfused with 4.0 ml. of Krebs-Ringer phosphate solution containing 2.0 ml. of rat gastric juice or other source of intrinsic factor while the other loop was perfused with a solution identical except that the source of intrinsic factor was replaced by an equal volume of water. In other experiments 0.5 ml. of human gastric juice or 1.0 ml. of a 1:20 dilution of the hog intrinsic factor preparation was substituted for the rat gastric juice.

Results. Inspection of Figure 1 shows that rat gastric juice clearly augmented the uptake of Co⁶⁰-B₁₂ by the intestinal wall. Augmentation by human gastric juice was less impressive and,

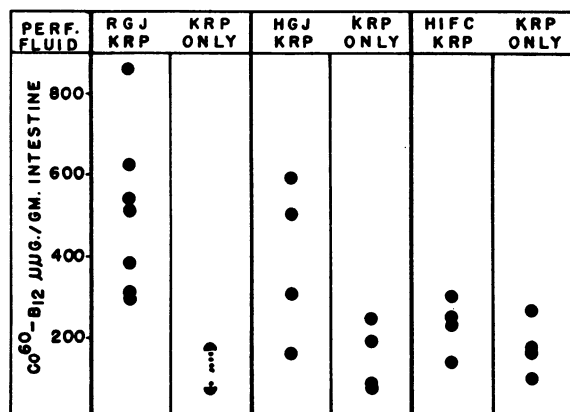


FIG. 1. EFFECT OF THREE DIFFERENT SPECIES OF INTRINSIC FACTOR GIVEN AS RAT GASTRIC JUICE (RGJ), HUMAN GASTRIC JUICE (HGJ) AND HOG INTRINSIC FACTOR CONCENTRATE (HIFC), RESPECTIVELY, UPON THE ADSORPTION OF Co⁶⁰-B₁₂ BY ONE OF A PAIR OF LOOPS OF RAT INTESTINE

Each circle represents the amount of Co⁶⁰-B₁₂ adsorbed during a single perfusion experiment with the species of intrinsic factor indicated (left-hand columns). The control value with Krebs-Ringer phosphate (KRP) solution alone in the other member of a given pair of loops is shown by one of the circles in the adjacent (right-hand) columns. Each perfusion fluid contained 0.015 μg. of Co⁶⁰-B₁₂. It appears that rat gastric juice is active, human gastric juice less so, and the hog intrinsic factor preparation not at all.

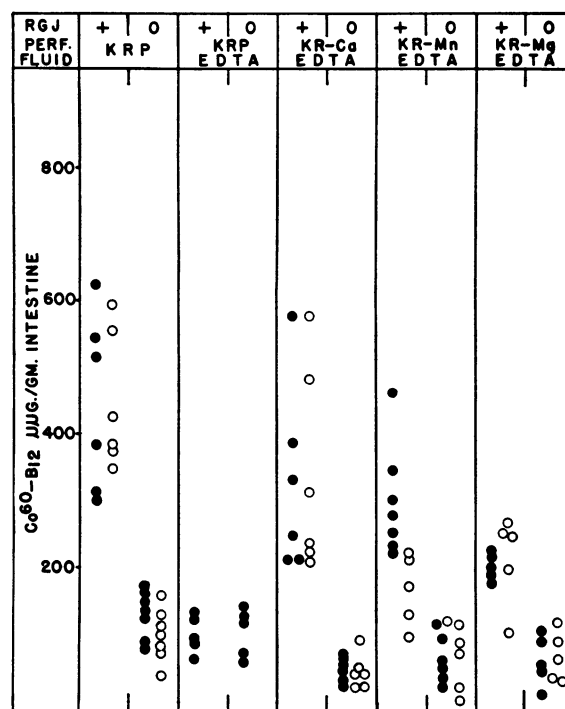


FIG. 2. EFFECT OF CALCIUM LACK AND OF SUBSTITUTION OF MANGANESE AND OF MAGNESIUM IONS FOR CALCIUM IONS UPON THE ADSORPTION OF Co⁶⁰-B₁₂ WITH (LEFT-HAND COLUMNS) AND WITHOUT (RIGHT-HAND COLUMNS) RAT GASTRIC JUICE (RGJ)

Each circle represents the amount of Co⁶⁰-B₁₂ adsorbed by half of each member of a pair of loops of rat intestine. After the perfusion, half of each loop was rinsed in Krebs-Ringer phosphate (KRP) solution (solid circles), the other half loop in disodium ethylenediaminetetraacetate (EDTA) solution (open circles). Thus, in each experiment the adsorbed radioactivity of each half of each pair of loops was measured; in all, four specimens. It appears that calcium is required for the adsorption of Co⁶⁰-B₁₂ under the influence of rat gastric juice and that manganese and perhaps magnesium may partially substitute for calcium ions.

as shown in Figure 3, with twice the amount of gastric juice was even less. With the hog intrinsic factor preparation, enhancement was undetectable.

Effect of calcium and other bivalent cations on uptake of Co⁶⁰-B₁₂. Suggested by the work of Herbert (14, 25), studies were made concerning the effect of bivalent cations. Special Krebs-Ringer phosphate solutions were prepared in which disodium ethylenediaminetetraacetate (EDTA) replaced sodium chloride in the initial rinsing fluid, as well as in the perfusion fluid. In some

experiments the standard, and in others the special EDTA-containing, Krebs-Ringer phosphate solution was employed in both loops, while in one loop the perfusion solution contained 2.0 ml. of neutralized rat gastric juice and the other contained an equal added volume of water. After the perfusion each intestinal loop was removed from the animal and everted on a metal rod. Then, in all the experiments except those in which the perfusion fluid contained sodium EDTA, the loop was carefully divided in half longitudinally with a safety razor blade, while still on the rod. Each half-loop was then washed in Krebs-Ringer phosphate solution for five minutes, the other half in an aqueous solution containing 100 mMoles per L. of sodium EDTA at pH 7.0. Thereafter, the radioactive content of each loop or half-loop was determined as usual.

Other special Krebs-Ringer solutions were prepared in which the EDTA was balanced by an equivalent amount of calcium (as CaCl_2), manganese (as MnSO_4), or magnesium (as MgSO_4). Phosphate was omitted from these solutions in order to prevent precipitation, and they were adjusted to pH 7.5 with 2 N NaOH. The EDTA exerted sufficient buffering power to maintain the pH of the effluent from the intestinal loop between 7.0 and 7.5 as determined by nitrazine paper. Both intestinal loops were initially rinsed and perfused in different experiments with either the standard or a special Krebs-Ringer solution. After the completion of the perfusion, half of each loop was rinsed in the standard Krebs-Ringer phosphate solution and half in the sodium EDTA solution. Finally the radioactivity of each half-loop was determined in the usual fashion.

Results. As shown in Figure 2 augmentation of $\text{Co}^{60}\text{-B}_{12}$ uptake by rat gastric juice in Krebs-Ringer phosphate solution was not affected by rinsing the intestinal segment with aqueous sodium EDTA solution *after* the perfusion. When, however, EDTA was incorporated into the pre-perfusion rinse and into the perfusion fluid containing rat gastric juice, there was abolition of the enhanced uptake of $\text{Co}^{60}\text{-B}_{12}$. The use of perfusion and pre-perfusion rinsing fluids containing calcium EDTA again resulted in an enhanced uptake of $\text{Co}^{60}\text{-B}_{12}$ by the intestinal wall in the presence of rat gastric juice. This radioactivity was also not removed from the half-loops

washed with sodium EDTA *after* the perfusion. The use of manganous EDTA during perfusion also allowed enhanced radioactive uptake by the intestine in the presence of rat gastric juice, but a portion of this radioactivity was removed by the subsequent rinsing of the half-loops of intestine with sodium EDTA. The presence of magnesium EDTA in the perfusion fluids resulted in a slight increase of radioactivity by the intestine under the influence of rat gastric juice, but the increase was small and its subsequent elution by sodium EDTA uncertain. In dialysis experiments (not shown) sodium EDTA did not bind vitamin B_{12} against dialysis, and had no effect on the dialyzability of $\text{Co}^{60}\text{-B}_{12}$ bound to rat stomach extract.

Effect of time of perfusion on uptake of $\text{Co}^{60}\text{-B}_{12}$. Paired loops of rat ileum were perfused in single experiments with identical amounts of Krebs-Ringer phosphate solution containing 1.0 ml. of rat gastric juice and 0.015 $\mu\text{g.}$ of $\text{Co}^{60}\text{-B}_{12}$ but for different periods of time. In the completed series of 27 experiments perfusion times ranged from 4 to 34 minutes. The perfusions were begun at different times and carried out at different rates in each loop so that perfusion of both loops was completed simultaneously. Thereafter each loop was removed, everted and divided in half longitudinally. One half was then rinsed in Krebs-Ringer phosphate solution and the other half in sodium-EDTA solution.

An identical procedure was carried out using 0.5 ml. of pooled human gastric juice (a quantity saturated by 0.015 $\mu\text{g.}$ of $\text{Co}^{60}\text{-B}_{12}$). In other experiments 1.0 ml. of human gastric juice (representing an excess of vitamin B_{12} binding power) was used. The effect of time of perfusion and of the subsequent rinsing of one of the half-loops of intestine in Krebs-Ringer phosphate solution and the other half in sodium EDTA solution was determined as in the experiments with rat gastric juice.

Results. Figure 3 demonstrates the linear increase with time of the average amounts of $\text{Co}^{60}\text{-B}_{12}$ adherent to loops of rat ileum after perfusion with solutions containing rat or human gastric juice, respectively. The values for each experiment, likewise expressed as micromicrograms of vitamin B_{12} and obtained by multiplying the data for radioactivity by a conversion factor,

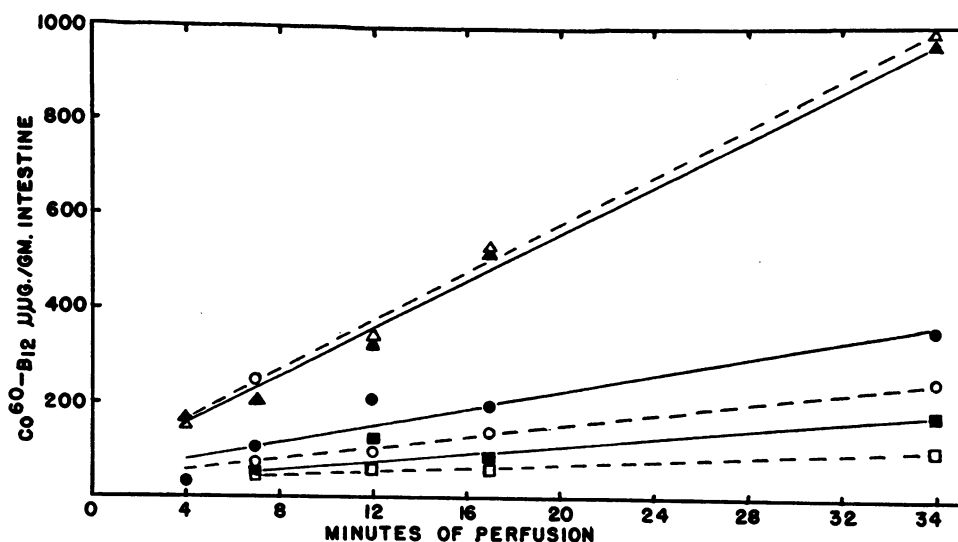


FIG. 3. EFFECT OF TIME OF PERFUSION, OF SPECIES OF GASTRIC JUICE AND OF SUBSEQUENT RINSING WITH DISODIUM ETHYLENEDIAMINETETRAACETATE (EDTA) SOLUTION UPON THE AVERAGE AMOUNTS OF Co⁶⁰-B₁₂ ADSORBED BY PAIRED LOOPS OF RAT INTESTINE

The data from individual experiments upon which the average values plotted in the figure are based are shown in Table III. Solid symbols and solid lines indicate average values after rinsing with Krebs-Ringer phosphate (KRP) solution; open symbols and dashed lines indicate average values after rinsing with EDTA solution. Results obtained with rat gastric juice saturated with Co⁶⁰-B₁₂ are shown by triangles, after similarly saturated human gastric juice by circles, and after half-saturated human gastric juice by squares. It is apparent that the adsorption of Co⁶⁰-B₁₂ increases in a linear fashion with time but more rapidly with rat than with human gastric juice. Note that the Co⁶⁰-B₁₂ adsorbed under the influence of rat gastric juice could not be removed by subsequent rinsing with EDTA solution, whereas with human gastric juice the adsorbed Co⁶⁰-B₁₂ could be partially removed by EDTA solution.

are listed in Table III. In every animal the intestinal loop perfused for the longer interval retained more radioactivity after rinsing than did the loop perfused for a shorter interval. When both kinds of gastric juice were saturated with respect to their vitamin B₁₂ binding capacity, the increase of radioactivity was much more rapid in the presence of rat than of human gastric juice. Moreover, when the same amount of Co⁶⁰-B₁₂, but twice the quantity of human gastric juice was perfused, the increase in radioactivity of the intestinal wall was still slower. Rinsing half of the intestinal loop with sodium EDTA after the experiment had no effect on the residual radioactivity after perfusion with solutions containing rat gastric juice but sharply reduced the radioactivity incorporated under the influence of human gastric juice.

Metabolic requirements of the uptake of Co⁶⁰-B₁₂. Perfusion studies were carried out using 2.0 ml. of rat gastric juice in Krebs-Ringer phosphate

solution in one loop and no gastric juice in the same perfusion fluid in the other. In order to determine the effect of ischemia (or anoxia) on the system, just before the initial pre-perfusion rinse with Krebs-Ringer phosphate solution the vessels at the root of the mesentery of the intestine were ligated.

Similar experiments were performed using loops with intact circulation as usual but with metabolic inhibitors added to the pre-perfusion rinsing and perfusion fluids in both loops. Sodium azide in a concentration of 10⁻² M was chosen because the animals survived this concentration for the duration of the experiment. When double this concentration of azide was used all of the animals died three to five minutes before the end of the perfusion. Perfusion solutions containing 10⁻² M sodium cyanide were lethal to most of the rats during the perfusion. In other experiments 10⁻² M dinitrophenol was employed.

TABLE III

*Effect of time of perfusion, species of gastric juice and of subsequent rinsing with disodium ethylenediaminetetraacetate upon the amount of $\text{Co}^{60}\text{-B}_{12}$ adsorbed by paired loops of rat intestine following perfusion of the same amount of $\text{Co}^{60}\text{-B}_{12}$ through each loop**

Perfusion mixture	Postperfusion rinsing solution	Time of perfusion				
		4 min.	7 min.	12 min.	17 min.	34 min.
Rat gastric juice, 1.0 ml., and 0.015 $\mu\text{g. Co}^{60}\text{-B}_{12}$ in 4 ml. of KRP	KRP†	286‡	245	490	624	1,480
	KRP	203	143	400	547	1,050
	KRP	132	222	361	300	475
	KRP	78		206	810	840
	KRP	99		146	512	
	KRP				312	
	Av.	160	203	321	518	961
	EDTA§	258‡	208	536	558	1,370
	EDTA	181	136	428	593	1,125
	EDTA	99	407	332	378	482
	EDTA	95		184	915	950
	EDTA	140		234	377	
	EDTA				350	
	Av.	154	250	343	529	982
Human gastric juice, 0.5 ml., and 0.015 $\mu\text{g. Co}^{60}\text{-B}_{12}$ in 4 ml. of KRP	KRP	36	51	198	216	333
	KRP	31	43	134	186	357
	KRP		252	366	198	267
	KRP		87	137	176	463
	Av.	34	108	208	194	355
	EDTA	41	31	72	184	229
	EDTA	23	37	92	110	229
	EDTA		155	126	139	194
	EDTA		78	95	120	330
	Av.	32	75	96	138	246
	KRP		43	123	186	179
	KRP		58	202	73	230
	KRP			55	46	122
	KRP				36	159
	KRP					176
	Av.		51	127	85	173
	EDTA		37	15	110	87
	EDTA		49	108	23	134
	EDTA			55	62	149
	EDTA				50	69
	EDTA					73
	Av.		43	59	61	102
Human gastric juice, 1.0 ml., and 0.015 $\mu\text{g. Co}^{60}\text{-B}_{12}$ in 4 ml. of KRP	KRP					
	KRP					
	KRP					
	KRP					
	KRP					
	Av.					
	EDTA					
	EDTA					
	EDTA					
	EDTA					
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	EDTA					
	EDTA					
	Av.					

* The values shown are for adsorbed $\text{Co}^{60}\text{-B}_{12}$ (micromicrograms per gram of intestine).

† Krebs-Ringer phosphate solution.

‡ The values for corresponding half-loops of rat intestine after rinsing with KRP and EDTA, respectively, are shown in corresponding positions in each column of figures.

§ Disodium ethylenediaminetetraacetate solution.

Results. As shown in Figure 4, ligation of the mesenteric vessels of the intestinal loop abolished the action of rat gastric juice. On the contrary, the presence of 10^{-2} M sodium azide in the pre-perfusion rinsing and perfusion fluids did not affect the action of rat gastric juice. Doubling

this concentration resulted in death of all the animals during the perfusion period, but nevertheless a significant augmentation of uptake of $\text{Co}^{60}\text{-B}_{12}$ was produced by the rat gastric juice. Consequently, it was assumed that even at the lower concentration a lethal concentration of azide was

probably presented to the intestinal mucosal cells. Sodium cyanide and dinitrophenol were also not inhibitory (data not shown).

Phase III. Release by the intestinal wall of vitamin B₁₂ bound to intrinsic factor

Because vitamin B₁₂ in high dosage may be absorbed in man (16, 17, 26) without the assistance of intrinsic factor, it was conceived that release of vitamin B₁₂ concentrated at the intestinal wall by intrinsic factor might represent the next physiological step in its assimilation. Consequently, dialysis was carried out with cellophane membranes as described in the section on Phase I in order to determine whether and under what conditions an aqueous extract of rat intestine would make dialyzable Co⁶⁰-B₁₂ initially bound to intrinsic factor.

Equivalence of binding capacities of different species of intrinsic factor preparations. As a preliminary to such experiments it was necessary to determine the amounts of each intrinsic factor preparation possessing similar binding capacities for Co⁶⁰-B₁₂. Accordingly, increasing amounts of Co⁶⁰-B₁₂ up to 0.06 μ g. per ml. were added to rat stomach extract, to human gastric juice and to the hog intrinsic factor preparation when suitably diluted. These preparations were then dialyzed individually in 0.0125 M phosphate buffer at pH 7.0 and 37° C. for five hours. It was assumed that the Co⁶⁰-B₁₂ crossing the cellophane membrane was balanced by an equal quantity of dialyzable vitamin which had not crossed the membrane. The bound vitamin thus was calculated for each sample as the difference between the total concentrations of Co⁶⁰-B₁₂ on opposite sides of the membrane.

Results. In Figure 5 are shown the quantities of Co⁶⁰-B₁₂ bound by 1 ml. of each of the intrinsic factor preparations: rat stomach extract, pooled human gastric juice and the hog intrinsic factor preparation diluted 1:16 in water. As may be seen, their binding capacities were approximately equal and increased in direct proportion to the amount of Co⁶⁰-B₁₂ added up to about 0.02 μ g. per ml. With larger amounts the primary binding capacity was apparently exceeded and the amounts of Co⁶⁰-B₁₂ bound were distinctly less than the amount added.

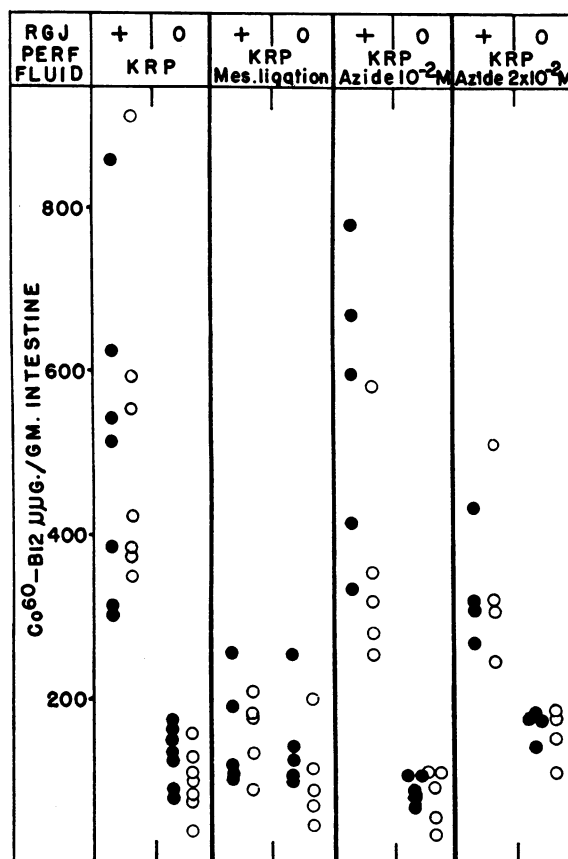


FIG. 4. EFFECT OF LIGATION OF THE MESENTERIC VESSELS, OF SODIUM AZIDE AND OF SUBSEQUENT RINSING WITH DISODIUM ETHYLENEDIAMINETETRAACETATE (EDTA) SOLUTION UPON THE ADSORPTION OF Co⁶⁰-B₁₂ WITH (LEFT-HAND COLUMN) AND WITHOUT (RIGHT-HAND COLUMN) RAT GASTRIC JUICE (RGJ)

Each circle represents the amount of Co⁶⁰-B₁₂ adsorbed by half of each member of a pair of loops of rat intestine. After the perfusion, half of each loop was rinsed in Krebs-Ringer phosphate (KRP) solution (solid circles) and the other half with EDTA solution (open circles). Thus, in each experiment the adsorbed radioactivity of each half of each pair of intestinal loops was measured; in all, four specimens. It is apparent that interruption of the blood supply but not the presence of sodium azide prevents the adsorption of Co⁶⁰-B₁₂ under the influence of rat gastric juice. Once so adsorbed, EDTA solution apparently does not remove it.

Effects of rat intestinal extract on release of Co⁶⁰-B₁₂ bound to different species of intrinsic factor preparations with similar binding capacities. Paired aliquots of rat stomach extract, of human gastric juice and of the diluted hog intrinsic factor preparation, respectively, to which increasing

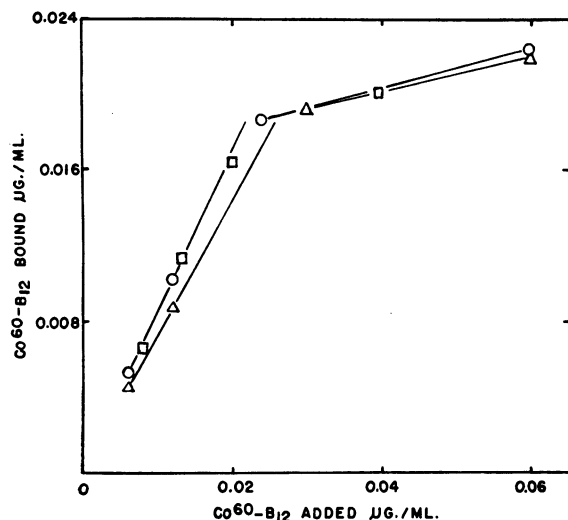


FIG. 5. COMPARATIVE AMOUNTS OF $\text{Co}^{60}\text{-B}_{12}$ BOUND BY RAT STOMACH EXTRACT (TRIANGLES), HUMAN GASTRIC JUICE (CIRCLES) AND HOG INTRINSIC FACTOR PREPARATION SUSPENDED IN 16 PARTS OF WATER (SQUARES)

To a constant amount of each was added increasing amounts of $\text{Co}^{60}\text{-B}_{12}$. After the usual dialysis procedure the $\text{Co}^{60}\text{-B}_{12}$ remaining bound by each sample was determined. The similar and linear relation for each preparation between the $\text{Co}^{60}\text{-B}_{12}$ bound and that added is apparent up to about 0.02 $\mu\text{g. per ml.}$ of $\text{Co}^{60}\text{-B}_{12}$.

amounts of $\text{Co}^{60}\text{-B}_{12}$ had been added up to 0.06 $\mu\text{g. per ml.}$ were set up. To one member of each pair was added a constant amount of rat intestinal extract, to the other was added a similar amount of water. Each pair was then dialyzed in 0.0125 M phosphate buffer at pH 7.0 and 37° C. for five hours. The differences between the $\text{Co}^{60}\text{-B}_{12}$ remaining bound in the individual pairs of dialysis tubes were taken to represent the quantities of $\text{Co}^{60}\text{-B}_{12}$ made dialyzable by the intestinal extract. In other experiments, $\text{Co}^{60}\text{-B}_{12}$ was added to the intestinal extract in the absence of intrinsic factor preparations and the binding capacity of the intestinal extract alone was estimated from the quantity of radioactivity that passed the cellophane membrane compared to that migrating in phosphate buffer alone.

Results. As shown in Figure 6, in experiments employing these preparations, the vitamin B_{12} made dialyzable from rat stomach extract by the subsequent addition of equal quantities of rat intestinal extract was 10 times that made dialyzable from human gastric juice. None of the vitamin was consistently made dialyzable from the

hog intrinsic factor preparation by rat intestinal extract (data not shown).

The native vitamin B_{12} content of rat intestinal extract, as determined microbiologically, was only 0.025 $\mu\text{g. per ml.}$ Alone it had little or no binding power for added $\text{Co}^{60}\text{-B}_{12}$ *in vitro*. Thus, 1.0 ml. of the extract was found to allow from 47 to 50 per cent of 0.006 $\mu\text{g.}$ of added $\text{Co}^{60}\text{-B}_{12}$ to dialyze in five hours at 37° C. A similar result occurred with an equal concentration of $\text{Co}^{60}\text{-B}_{12}$ in Krebs-Ringer phosphate buffer alone.

Kinetics of the release of $\text{Co}^{60}\text{-B}_{12}$ from rat stomach extract by rat intestinal extract. In other experiments a constant amount of rat in-

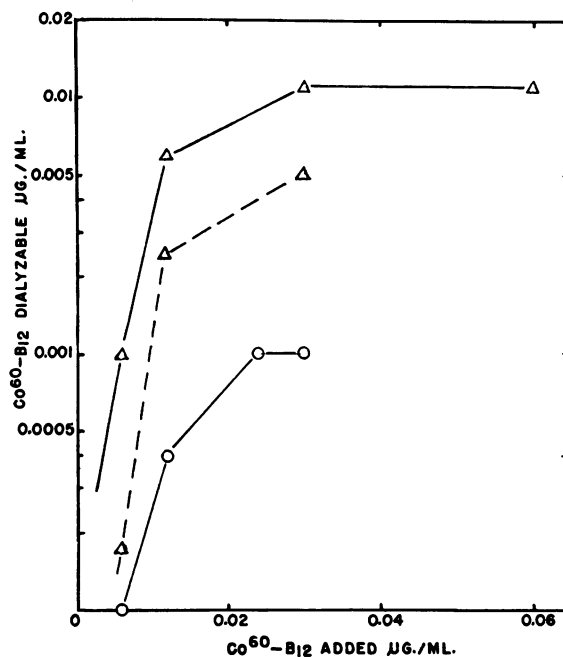


FIG. 6. COMPARATIVE EFFECTS OF A CONSTANT AMOUNT OF RAT INTESTINAL EXTRACT IN RENDERING DIALYZABLE $\text{Co}^{60}\text{-B}_{12}$ BOUND TO RAT STOMACH EXTRACT (TRIANGLES) AND TO HUMAN GASTRIC JUICE (CIRCLES), RESPECTIVELY

As shown in Figure 5, these preparations as well as a preparation of hog intrinsic factor concentrate had similar binding capacities for $\text{Co}^{60}\text{-B}_{12}$. The effect of previously boiled rat intestinal extract upon $\text{Co}^{60}\text{-B}_{12}$ bound to rat stomach extract is also shown (triangles connected by dashed line). Semilog scales are used. It appears that the same amount of rat intestinal extract is able to render dialyzable about 10 times as much $\text{Co}^{60}\text{-B}_{12}$ bound to rat stomach extract as when bound to human gastric juice. The intestinal extract was still active after boiling. However, even unboiled extract had no effect upon $\text{Co}^{60}\text{-B}_{12}$ bound to the hog intrinsic factor preparation (results not shown).

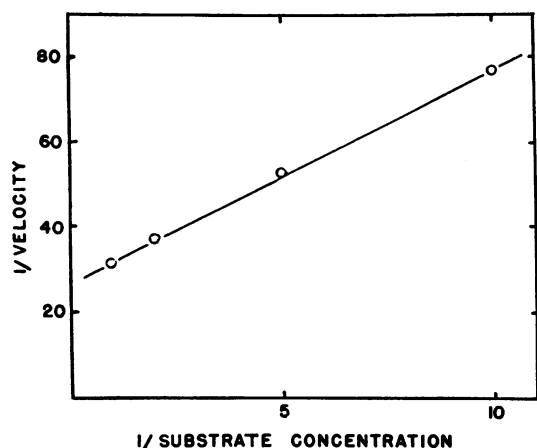


FIG. 7. AMOUNT OF Co⁶⁰-B₁₂ RENDERED DIALYZABLE FROM VARIOUS CONCENTRATIONS OF RAT STOMACH EXTRACT DURING INCUBATION AT 37° C. FOR FIVE HOURS WITH A CONSTANT QUANTITY OF RAT INTESTINAL EXTRACT

The reciprocal of the Co⁶⁰-B₁₂ rendered dialyzable (velocity of the reaction) is plotted on the vertical axis while the reciprocal of the concentration of the rat stomach extract (the substrate) is plotted on the horizontal axis according to the method of Lineweaver and Burk (27). A linear relation is evident.

testinal extract was incubated during the usual dialysis period of five hours at 37° C. with various amounts of rat stomach extract previously saturated with Co⁶⁰-B₁₂. The quantity of Co⁶⁰-B₁₂ made dialyzable by the intestinal extract was measured.

Results. In Figure 7 are plotted the quantities released from 1.0, 0.5, 0.2 and 0.1 ml., respectively, of rat stomach extract by a constant quantity of rat intestinal extract during five hours under the above conditions. These were arbitrarily taken as a measure of the velocity of the reaction according to the method of Lineweaver and Burk (27). The linear relationship obtained suggests that the five hour incubation interval chosen was probably within the period of constant velocity of the reaction, and also that kinetics of the reaction may be described by the mass law and by the Michaelis-Menten equation. Such kinetics are characteristic of enzyme reactions, but do not prove this is necessarily the case here.

Effect of previous boiling, low pH, and metabolic inhibitors on the activity of rat intestinal extract. Rat intestinal extract was boiled on a water bath for 20 minutes. Then to a constant amount

of rat stomach extract were added increasing amounts of Co⁶⁰-B₁₂ up to 0.06 µg. per ml. These mixtures were then dialyzed as usual in the presence and in the absence of a constant amount of the boiled rat intestinal extract and the Co⁶⁰-B₁₂ rendered dialyzable by the intestinal extract was measured.

The effect of pH on the action of unboiled rat intestinal extract was also determined by dialysis. Equal quantities of rat stomach extract saturated by 0.006 µg. of Co⁶⁰-B₁₂ per ml. were dialyzed at pH 3.0, 4.0 and 7.5, respectively, in the presence and in the absence of a constant quantity of rat intestinal extract. The pH of each system was adjusted with 2 N hydrochloric acid before dialysis. After dialysis the amounts of Co⁶⁰-B₁₂ still bound at each pH in the presence and in the absence of intestinal extract were compared as a measure of its activity.

Measured amounts of rat intestinal extract were briefly incubated with 1.2×10^{-2} M mercuric chloride and 1.2×10^{-2} M sodium arsenite. Thereafter, similar quantities of rat stomach extract saturated by 0.006 µg. of Co⁶⁰-B₁₂ were incubated during the usual dialysis period with equal amounts of each of the two preparations of rat intestinal extract containing the metabolic inhibitors as well as with an unmodified sample. The amounts of Co⁶⁰-B₁₂ released by each preparation of rat intestinal extract were determined.

Results. Boiling the rat intestinal extract reduced, but did not abolish, its ability to make dialyzable a portion of the vitamin B₁₂ bound to rat stomach extract (Figure 6). Acidification of the system prevented most of the Co⁶⁰-B₁₂ bound by rat stomach extract from being made dialyzable by rat intestinal extract (Table IV). As

TABLE IV

Effect of acidity on the activity of rat intestinal extract in making dialyzable Co⁶⁰-B₁₂ bound to rat stomach extract

Rat stomach extract containing Co ⁶⁰ -B ₁₂ (0.006 µg. per ml.)	Rat intestinal extract	pH	Co ⁶⁰ -B ₁₂ bound after dialysis µg. per ml.
+	0	7.5	4,882
+	+	7.5	1,930
+	+	4.0	3,762
+	+	3.0	4,274

shown in Table V, the presence of mercuric chloride or of sodium arsenite in the quantities used resulted in much of the $\text{Co}^{60}\text{-B}_{12}$ remaining bound to the rat stomach extract.

Characteristics of the dialyzable radioactivity. The dialysate obtained after incubation of rat stomach extract saturated with $\text{Co}^{60}\text{-B}_{12}$ together with rat intestinal extract was diluted in 0.05 M phosphate buffer at pH 7.0 and shaken with an equal volume of benzyl alcohol. The partition coefficient of the radioactivity for benzyl alcohol over water was determined as 0.75 to 0.77, which compares closely with that of $\text{Co}^{60}\text{-B}_{12}$. This suggests that the dialyzed material is free cyanocobalamin, since different distribution ratios in the solvents would be expected if a fragment of intrinsic factor or other organic substance was bound to the vitamin.

Other characteristics of rat intestinal extract. Rat intestinal extract was incubated with 1 per cent dextran solution for 90 minutes at 37°C . and pH 6.0. The reducing activity of the system (determined as glucose) at 30 minute intervals gradually rose (Table VI). Rat intestinal extract was also incubated with an equal volume of 1 per cent soluble starch solution at pH 6.0 at 37°C . The intensity of the blue color produced by the addition of iodine, as determined at intervals in a Klett-Summerson photoelectric colorimeter with a red filter (No. 1—640 to 700 $\text{m}\mu$.), fell rapidly (Table VII).

The intestinal extract thus was capable of hydrolyzing dextran and had amylase-like properties.

DISCUSSION

The dialysis experiments described indicate that human gastric juice is capable of competitively

TABLE V

Effects of metabolic inhibitors on the activity of rat intestinal extract in making dialyzable $\text{Co}^{60}\text{-B}_{12}$ bound to rat stomach extract

Rat stomach extract containing $\text{Co}^{60}\text{-B}_{12}$ (0.006 μg . per ml.)	Rat intestinal extract	Inhibitors	$\text{Co}^{60}\text{-B}_{12}$ bound after dialysis μg . per ml.
+	0	0	4,280
+	+	0	2,220
+	+	HgCl_2	3,720
+	+	NaAsO_2	3,340

TABLE VI

Effect of incubation of rat intestinal extract on the reducing power of dextran solution at 37°C . and pH 6.0

Time of incubation	Reducing power (as glucose)
min.	mg./100 ml.
0	86.8
30	97.1
60	114.0
90	123.4

removing native vitamin B_{12} from beef and pork muscle (Table II), and of removing added radioactive vitamin B_{12} from these substances as well as from casein (Table I). Because vitamin B_{12} bound to gastric juice is preferentially absorbed both in pernicious anemia (12) and in the intestinal loop of the gastrectomized rat (13), such binding may constitute an essential initial step in the action of intrinsic factor with respect to the relatively small amounts of vitamin B_{12} in most natural food sources. Because vitamin B_{12} diffuses more readily from food proteins at an acid pH but is bound equally well by human gastric juice or hog gastric mucosal extract (28) at neutral or acid pH values, the presence of normal gastric acidity would appear to be of physiologic advantage in the postulated Phase I of the action of intrinsic factor.

In the rat experiments with paired intestinal loops with intact blood supply, the presence of bivalent cations—especially Ca^{++} —appeared to be necessary for the uptake of vitamin B_{12} by the rat intestine under the influence of rat gastric juice (Figure 2). Thus, rat gastric juice was ineffectual in enhancing the uptake of $\text{Co}^{60}\text{-B}_{12}$ when Ca^{++} was chelated by EDTA during the perfusion. Because it was shown in dialysis experiments that EDTA neither bound $\text{Co}^{60}\text{-B}_{12}$ nor affected its dialyzability when bound to rat stomach extract, the effect of EDTA in the intestinal loop must have been on the uptake of $\text{Co}^{60}\text{-B}_{12}$ already bound to rat intrinsic factor. Chelating the Ca^{++} with EDTA after the perfusion did not remove the radioactivity from the intestinal wall. This suggests that the intrinsic factor-bound $\text{Co}^{60}\text{-B}_{12}$ first adheres to the intestinal wall by means of a calcium-containing bond, but that later this bond to the intestine is dissolved or ren-

TABLE VII

Effect of incubation of rat intestinal extract on soluble starch solution at 37° C. and pH 6.0

Time of incubation	Blue color with iodine
<i>min.</i>	<i>Klett units</i>
0	240
3	198
5	14
7	0
9	0

dered unavailable to EDTA. The ease with which the radioactivity may be removed from the intestinal wall when calcium is replaced by manganese suggests that manganese is less efficient than calcium as a bivalent cation in the binding process (Figure 2). This process, so defined, is postulated as Phase II of the action of intrinsic factor.

It is known (29) that a number of mucoproteins contain bonds consisting of $(\text{CaSO}_4)^+$ complexes linking amino groups and carboxyl groups, and that this bond may be broken with EDTA. Monovalent cations may replace calcium in this type of bond with marked change in the physical properties of the mucoprotein. Since intrinsic factor is thought to be a mucoprotein (30, 31), this might explain its functional need for bivalent cations. Miller and Hunter (32) and Herbert (25) have demonstrated that hog intrinsic factor will bind $\text{Co}^{60}\text{-B}_{12}$ to rat liver slices. Moreover, Herbert (25) has found that calcium is required for this effect and that much of this radioactivity may subsequently be removed with EDTA. Calcium has also been shown to be essential for the uptake of $\text{Co}^{60}\text{-B}_{12}$ by everted segments of rat intestine (14) and by the human intestine (33, 34). Inhibition without complete destruction of human (35) and of rat (13) intrinsic factor activity by low pH has been reported. The marked reduction in ionization of carboxyl groups at low pH would be expected to prevent the formation of the $\text{NH}_2\text{-SO}_4\text{-Ca-OOC}$ complex. With experimentally maintained acidity of the intestinal contents this would occur at the mucosal surface but not within the wall of the living intestine.

If the calcium-dependent bond is accepted as essential to Phase II, the initial reaction between intrinsic factor and the intestinal wall in the pres-

ence of Ca^{++} , there still remains to be explained the inability of EDTA to remove $\text{Co}^{60}\text{-B}_{12}$ bound to the wall only a few minutes after the completion of the perfusion with rat gastric juice. This observation requires that by then the calcium bond is either shielded from the action of EDTA or that the $\text{Co}^{60}\text{-B}_{12}$ has already been transferred to another type of bond in which bivalent cations are not important. The likelihood of the second possibility becomes apparent from consideration of the action of human gastric juice in the experiments with rat intestinal loops. (Table III and Figure 3.) With human gastric juice the uptake of radioactivity is much slower and a portion of the radioactivity may be removed by subsequent rinsing with EDTA. Moreover, the fraction which cannot be removed by EDTA increases with the duration of the perfusion. Thus, the initial Ca^{++} -dependent bonding may be the essential preliminary step in Phase II, and the change of bond may be the rate-limiting factor in the subsequent absorption of vitamin B₁₂ during the hypothetical Phase III of the action of intrinsic factor.

The presence of 2×10^{-2} M sodium azide in the perfusing fluid was sufficient to kill the rat but still allowed an augmented uptake of radioactivity by the intestinal loop under the influence of rat gastric juice. Similar results were obtained with 10^{-2} M dinitrophenol (data not presented). It is probable, therefore, that 10^{-2} M sodium azide, which also caused no inhibition of uptake is sufficient to inhibit respiration in the intestinal mucosal cells. This suggests, as does the activity of hog intrinsic factor in everted sacs of rat intestine at 6° C. (14), that adsorption of vitamin B₁₂ to the rat intestine in Phase II is a physical process that is not species-related and that does not require the expenditure of energy. This is, of course, not to say the same of its further transport within the intestinal wall, for which Abels (36) has recently suggested pinocytosis at a molecular level as a possible initial mechanism.

In the dialysis experiments with a crude extract of rat intestine its 10-fold greater ability to release what appears to be free vitamin B₁₂ from rat stomach extract compared to its effect on human gastric juice corresponds with the differential effects of rat and of human intrinsic factor

preparations in promoting the assimilation of vitamin B₁₂ by the gastrectomized rat (37) and by the rat intestinal loop (Figure 1). Moreover, this species-related activity of rat intestinal extract, if concerned in the conversion of the Ca⁺⁺-dependent bond might explain the correspondingly greater ability of EDTA to remove a portion of the radioactivity from the intestine after perfusion with human gastric juice (Figure 3). EDTA was similarly effective in Herbert's experiments with a hog intrinsic factor preparation on surviving rat liver slices (25) and everted sacs of rat intestine (15). The total inability of the rat intestinal extract to free vitamin B₁₂ from the hog intrinsic factor preparation also resembles the complete failure of hog intrinsic factor preparations to enhance vitamin B₁₂ adsorption in either the gastrectomized rat or the rat intestinal loop. For this reason, conclusions concerning the effects of hog intrinsic factor preparations upon rat tissues may be applicable only to certain phases of its physiological action. However, such experiments have disclosed chemical circumstances essential to part of the physiological process (14, 25).

Without the aid of intrinsic factor, absorption of vitamin B₁₂ may be accomplished only by using relatively large doses of vitamin B₁₂. Since no convincing evidence exists that a vitamin B₁₂-peptide or other conjugate of vitamin B₁₂ with a small molecule is involved in the absorption of vitamin B₁₂ (38, 39), Phase III of the action of intrinsic factor may also include the destruction of the intrinsic factor-vitamin B₁₂ bond at or just within the intestinal wall, with consequent release of free cyanocobalamin. The vitamin B₁₂ thus freed would then either be accepted by an appropriate transport mechanism or would simply diffuse passively into the blood vessels of the intestine.

The active substance (or substances) in the rat intestinal extract reacts with rat stomach extract in a manner which follows the Lineweaver-Burk (27) variation of the Michaelis-Menten equation. This is compatible with enzymic activity, but does not prove that such is the case. The intestinal extract is partially inactivated by heat and hydrolyzes both starch and dextran. It may be identical with the dextran hydrolyzing enzyme described by Androuny, Bloom and Wilhelmi

(40) or with the 1,6-glycosidase described by Lerner and McNickle (41). No evidence, however, is available to prove that the active principle in this rat intestinal extract necessarily has any physiological significance with respect to vitamin B₁₂ absorption. It is interesting, however, that it exhibits a species-related activity similar to that of intrinsic factor in the rat.

SUMMARY

1. Human gastric juice competitively removes native vitamin B₁₂ or added Co⁶⁰-B₁₂ from pork or beef muscle preparations during dialysis *in vitro*. This process is favored by low pH because such food proteins, as well as casein, lose vitamin B₁₂ more readily at low pH values, whereas human gastric juice loses none of its native vitamin B₁₂ when dialyzed against water at either pH 6.0 or pH 1.5.

2. There is a linear increase with time in the quantity of Co⁶⁰-B₁₂ on or in the wall of loops of rat ileum with intact circulation after perfusion with rat or human intrinsic factor in the presence of Krebs-Ringer phosphate solution. This increase is more rapid in the presence of rat stomach extract than of human gastric juice. No certain effect was noted with a hog intrinsic factor preparation.

3. This action of preparations containing rat or human intrinsic factor is not affected by the presence of 2×10^{-2} M sodium azide, but is prevented by occluding the blood supply of the intestine. It is also prevented by the presence of sodium ethylenediaminetetraacetate (EDTA) during the perfusion but not by the presence of calcium EDTA. That the dialyzability of Co⁶⁰-B₁₂ alone or when bound to rat stomach extract is unaffected by sodium EDTA indicates that calcium is involved in the binding of intrinsic factor to the intestinal mucosa. Manganese or magnesium only partially neutralizes the inhibitory effect of sodium EDTA. A portion of the vitamin B₁₂ adsorbed by the rat intestine during perfusion with Krebs-Ringer phosphate in the presence of human gastric juice, but none of that adsorbed in the presence of rat stomach extract, is removed after the perfusion by rinsing with sodium EDTA.

4. A crude extract of rat intestine is capable of

making dialyzable, apparently as free vitamin, a portion of the Co⁶⁰-B₁₂ bound to rat stomach extract, much less of that bound to human gastric juice, and none of that bound to a hog intrinsic factor preparation. Its action is partially inhibited by boiling, by low pH and by mercuric chloride and sodium arsenite. The intestinal extract hydrolyzes both dextran and starch.

5. A hypothesis is presented to explain the observed differences in activity of different species of intrinsic factor in the absorption of vitamin B₁₂ by the gastrectomized rat. The present observations suggest that three phases can be recognized in the action of intrinsic factor in the rat: Phase I, a non-species-related competitive binding by intrinsic factor of dietary vitamin B₁₂; Phase II, a non-energy-requiring adsorption of intrinsic factor-bound vitamin B₁₂ by the intestinal mucosa involving a bivalent cationic dependent bond; and Phase III, a species-related conversion of the Ca⁺⁺-dependent bond and release of free vitamin B₁₂ at the surface of or within the intestinal wall, possibly as a result of intestinal enzymic action.

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