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Research Article

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IN VITRO ASSAY FOR HUMAN INTRINSIC FACTOR *

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More than three decades ago, it was discovered that normal human gastric juice contained an "intrinsic factor" lacking in the gastric juice of patients with pernicious anemia (1). Certain characteristics of this gastric intrinsic factor have been determined, such as its lability to heat (2) and to incubation below pH 3.5 (3) and its stability to moderately alkaline pH (4). Moreover, the function of intrinsic factor is inhibited by moderate acidity of the intestinal milieu (insufficient to destroy intrinsic factor) whether *in vivo* (5, 6) or *in vitro* (7).

Human intrinsic factor has not yet been isolated in pure form. A major hindrance in such efforts has been that the only reliable assay for intrinsic factor required the use of human subjects. Until 1952, this consisted in therapeutic trial, evaluated principally by comparative reticulocyte responses (8) in patients with untreated pernicious anemia. Subsequently, most assays have been performed by comparing the absorption of radioactive vitamin B_{12} fed alone and then with the intrinsic factor preparation to be assayed. This assay can be carried out on patients with either untreated or treated pernicious anemia and requires measurement of the amount of radioactivity in stool (9), urine (10), liver (11), plasma (12), or the whole body (13, 14). A simple *in vitro* assay for human intrinsic factor has long been sought.

Based on the finding (15) that vitamin B_{12} uptake by rat-liver slices was enhanced by hog intrinsic factor concentrate (HIFC), *in vitro* assays for hog intrinsic factor were reported using liver slices (16) and liver homogenates (17, 18). However, because of uncertain physiologic correlations, occasional failure of the systems to detect intrinsic factor in gastric juices containing free acid (19), relative insensitivity to human intrinsic factor, and the necessity of two separate incubations, these assays have not found wide use in the study of human intrinsic factor. Nevertheless, a liver homogenate assay procedure (20) has proved useful in helping to prepare one of the purest forms of commercial hog intrinsic factor concentrate yet available (21).

Wilson and Strauss (22, 23) and, subsequently, Wolff and Nabet (24) reported that vitamin B_{12} uptake by everted sacs of guinea pig ileum is facilitated by human intrinsic factor, and proposed this technique as an *in vitro* assay. Guinea-pig ileum is responsive to intrinsic factor from many species (22). Because of variations in the vitamin B_{12} uptake by different everted ileal sacs from the same animal, as well as because of differences between preparations from different animals, it was necessary for Wolff and Nabet to use one sac from each of ten guinea pigs for one human intrinsic factor assay (24). Boass and Wilson subsequently reported an assay for rat and hamster intrinsic factor, using many thin rings of hamster intestine (25) in each sample to reduce variability among samples.

The present study sought the advantage of using pooled lots of homogenized guinea-pig intestinal mucosa (7, 26) to circumvent the variabilities of the rat liver and guinea-pig ileum everted sac systems. This homogenate possesses these advantages: *1*) it eliminates variations in the activity of the mucosa of different intestinal segments from the same or from different animals; *2*) a single sample of homogenate can serve as a control for a number of experimental flasks; and *3*) large amounts of homogenate can be prepared and frozen in portions until used, eliminating the need for preparing material for each assay and making possible repeated assays with portions of the same substrate. The first two advantages,

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but not the third, may be obtained by using intact mucosal cells (27) or rings of intestinal mucosa (25, 28). Rings, however, introduce the problem of nonspecific serosal uptake (28). Although intact rat intestinal mucosal cells were found to be useful for assay of rat intrinsic factor and rings of hamster intestine were useful for assay of rat and hamster intrinsic factor, these systems were not successfully used to assay human intrinsic factor (25, 27, 28) as would be expected because of the relatively high degree of species specificity of these systems (22). The successful use of homogenates of guinea pig intestinal mucosa as an assay for human and hog intrinsic factor forms the basis of the present report.

MATERIALS AND METHODS

Co^{57}-labeled vitamin B_{12}. Different lots of Co^{57}-labeled vitamin B_{12} of varying specific activity (4.6 to 19.9 μc per μg) [1] were added to sufficient nonradioactive B_{12} and 0.9% NaCl, so that final concentration of $Co^{57}B_{12}$ was 10,000 picograms (pg) [2] per ml with specific activity varying from 0.04 μc per ml to 0.06 μc per ml. In our well-type scintillation counter, we obtained approximately 8 cpm per pg of $Co^{57}B_{12}$ of the lower specific activity. In the tables, the uptake of radioactive vitamin B_{12} has been expressed as picograms per portion of about 200 mg wet homogenate unless otherwise specified.

Guinea-pig small-intestinal mucosa homogenate. Nonfasting, 200- to 300-g, white, male guinea pigs were sacrificed in dozen lots. The small intestine, which averaged 180 cm in length, was transected at the pylorus, stripped free of its mesenteric attachment, and transected at its ileal end. The upper half of the small intestine was discarded, since the lower half exhibits the greatest degree of intrinsic factor-facilitated vitamin B_{12} uptake (23). The lower half was rinsed with 0.9% NaCl from a 100-ml syringe until the effluent was clear and free of particulate matter. It was then cut into segments 8 to 10 cm long. Each segment was placed on Parafilm, held at one end with forceps, and the mucosa was expressed by running a metal spatula along the length of the serosal surface while applying gentle pressure (30). The pooled mucosal scrapings from 12 guinea pigs (wet weight, 22 to 24 g) were added to 300 ml cold saline (25 ml per guinea pig) and homogenized for 30 seconds in a Waring Blendor. The homogenate suspension was pipetted into 24 equal samples in test tubes and centrifuged in the cold for 10 minutes at 2,000 $\times$ g. The supernatant fluid was discarded, and the homogenate pellet was kept at $-20°$ C until used.

Lyophilized, acetone-washed homogenate was prepared from the fresh mucosal homogenate obtained after centrifugation at 2,000 $\times$ g, by previously described lyophilization methods (20), and stored at room temperature until used. Approximately 1.5 to 1.7 g of lyophilized substance was obtained from each lot of 12 guinea pigs. Seven mg of lyophilized substance was used for each assay specimen.

Lyophilized homogenate-KRT buffer-B_{12} was prepared by mixing portions of lyophilized homogenate with Krebs-Ringer–Tris buffer, pH 7.4, containing 10 mM $CaCl_2$ (16), and $Co^{57}B_{12}$ in ratios of 7 mg: 5 ml: 0.5 ml (5,000 pg), respectively, and lyophilizing this mixture. For assay, 12 ml of deionized water was added to 2 portions of the lyophilized substance for each unknown sample.

National Formulary intrinsic factor concentrate reference standard (NFIF) (31) no. 6043 was obtained from the National Formulary of the American Pharmaceutical Association (1 test unit = 50 mg). For the *in vitro* system, 25 mg of this hog intrinsic factor concentrate was dissolved in 10 ml of 0.9% NaCl (125 μg NFIF per 0.05 ml saline) and stored at $-20°$ C until used.

Human gastric juices were obtained with continuous nasogastric suction under basal conditions and with augmented histamine stimulation in fasting subjects as described by Kay (32). Salivary contamination was minimized by having the subjects expectorate into a basin during the collection periods. Each specimen was measured and filtered through two layers of cheesecloth, and its pH was determined. Titration to pH 11 was accomplished with 10% potassium hydroxide to destroy peptic activity (4). After 20 minutes, the specimen was back-titrated to pH 7 with 0.1 N sulfuric acid and then stored at $-20°$ C until assayed.

In vitro assay system. Each sample of gastric juice or NFIF standard was assayed in duplicate in 20-ml Griffin beakers. For every six samples (three duplicates) one sample of frozen intestinal mucosa homogenate was thawed and reconstituted with 6 ml of 0.9% NaCl. Since two frozen samples of homogenate were made from the distal half of the small intestine of each guinea pig, material for 12 assay samples (six duplicate pairs) was derived from each animal.

To each beaker were added in sequence: 5 ml KRT buffer, 1 ml homogenate suspension (or 7 mg of lyophilized intestinal mucosa homogenate), the gastric juice to be assayed (0.1 ml, unless otherwise specified), and 0.5 ml $Co^{57}B_{12}$ solution (5,000 pg). In addition to the unknown gastric juices, duplicate 0.1-ml saline controls and 0.05 ml (125 μg)-NFIF standards were included in each group of assays. To assure uniformity of suspension of frozen homogenate after thawing, we agitated it for 3 to 5 seconds in a Waring Blendor after adding 6 ml of 0.9% NaCl per sample.

In studies of pH dependence, the buffer used consisted of 9 parts Krebs-Ringer solution (33), brought to appropriate pH with 1 part 0.05 M Tris-acid maleate-NaOH buffer (34).

[1] Kindly supplied by Dr. Elmer Alpert of Merck Sharp & Dohme Research Laboratories, West Point, Pa.

[2] One picogram (pg) = one micromicrogram ($\mu\mu$g) = 10^{-12} g. Prefix approved by International Union of Pure and Applied Physics (29).

All samples were gently agitated at a rate of 90 cycles per minute for 30 minutes at room temperature in a Dubnoff metabolic shaker. The homogenate was then precipitated by centrifugation at $2,000 \times g$ for 10 minutes. The supernatant fluid was discarded, after which the homogenate was suspended in, and centrifuged from, 10 ml and then 3 ml of 0.9% NaCl containing 10 mM $CaCl_2$. After the supernatant fluid was decanted, the radioactivity in the homogenate pellet was determined in a well-type scintillation detector. The counts per minute of duplicate samples were averaged and converted to picograms of B_{12}. Differences between duplicates were usually less than 10%.

Clinical (in vivo) assay system. The intrinsic factor activities of certain samples were determined in pernicious anemia patients, in remission, by the modification of the Schilling test adopted as standard by the National Formulary of the U.S.A. (31), using either 30 ml of the unknown gastric juice, or 50 mg of NFIF, and 2 μg of $Co^{60}B_{12}$.

RESULTS

A typical assay is shown in Table I. To correct for nonspecific uptake of B_{12} into the homogenate, a saline control was included. There was a fourfold or greater enhancement of B_{12} uptake in this experiment by intrinsic factor, as shown by the last column. Gastric juice from a patient with pernicious anemia did not enhance B_{12} uptake. The NFIF was included as a reference standard for the *in vitro* activity of the unknown human gastric juices by analogy with its similar use as a reference standard for the *in vivo* activity of commercial hog intrinsic factor concentrates (31).

Reproducibility of assay. In an experiment consisting of 20 assays of equal samples of the same normal gastric juice, the average counts per minute for the group was 202, with 19.6 SD, and a coefficient of variation of 9.7 (data not shown). The average of the saline controls was 36 cpm, with 4 SD. In a series of six different assays consisting of four samples of the same normal gastric juice, the coefficients of variation were 2.8, 1.8, 3.3, 3.0, 6.9, and 5.2, respectively. The variability between assays affected the NFIF and saline controls in parallel fashion, so that the ratio of B_{12} uptake of sample to B_{12} uptake of control showed less variation than did the absolute number of picograms of B_{12} taken up by different homogenates.

Effect of concentration of intrinsic factor. To determine a reliable concentration of gastric juice for use in the assay system, various amounts of gastric juice from normal volunteers, patients with normal serum B_{12} levels, and patients with pernicious anemia were assayed, as were various concentrations of NFIF. Figure 1 records the results of several experiments. Increasing amounts of normal gastric juice, up to 0.1 ml, resulted in increased B_{12} uptake by the homogenate. In the three specimens assayed in concentrations greater than 0.1 ml, there was only slightly greater enhancement at 0.2 ml in two specimens and a slight decline in B_{12} uptake in the third specimen. At 0.3 ml, the enhancement was identical to that obtained with 0.2 ml of gastric juice. Increased amounts of NFIF up to 0.05 ml caused increased B_{12} uptake, which reached a plateau between 0.05 and 0.5 ml and fell moderately at 1.0 ml. This reduced effect of excess intrinsic factor has previously been noted in liver slices (16) and everted sacs of rat small intestine (17). Concentrations of gastric juice and NFIF of 0.1 ml and 0.05 ml (125 μg), respectively, were selected for routine use on the basis of the findings noted in Figure 1.

TABLE I

Standard assay for human and hog intrinsic factor activity with guinea-pig intestinal mucosa homogenate

Source of intrinsic factor	B_{12} Uptake by homogenate*	$\frac{B_{12}\text{ Uptake of sample}}{B_{12}\text{ uptake of control}}$
	pg	
Normal volunteer†	72	4.5
Pernicious anemia patient†	17	1.1
Folic acid-deficient patient†	59	3.7
NFIF‡	80	5.0
Saline control	16	1.0

* Vitamin B_{12}, 5,000 picograms (pg), and 300 mg homogenate added to each beaker.
† Gastric juice, 0.1 ml.
‡ National Formulary hog intrinsic factor concentrate reference standard, lot 6043, 125 μg.

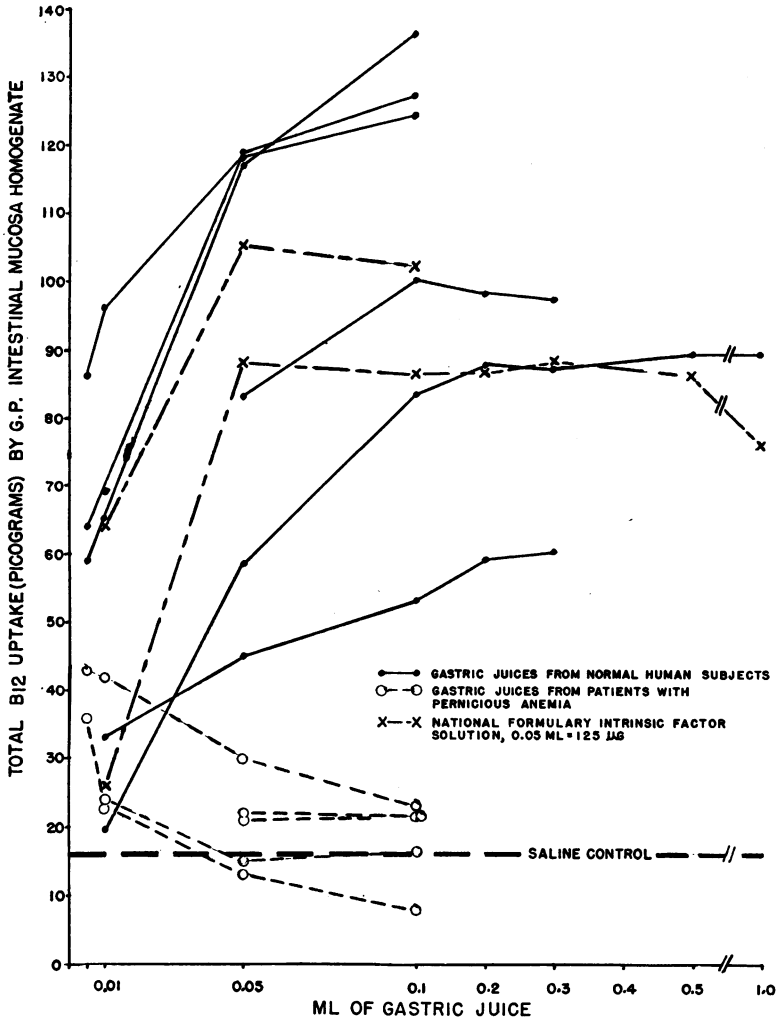

FIG. 1. EFFECT OF VARIOUS CONCENTRATIONS OF INTRINSIC FACTOR ON UPTAKE OF $Co^{57}B_{12}$ BY GUINEA-PIG INTESTINAL MUCOSA HOMOGENATE.

TABLE II

Binding capacity, determined by dialysis, of NFIF for varying concentrations of $Co^{57}B_{12}$

Outer solution	Inner solution	Percentage of $Co^{57}B_{12}$ bound by 125 µg NFIF
		%
5,000 pg B_{12} + 125 µg NFIF in 4 ml H_2O*	4 ml H_2O*	88
10,000 pg B_{12} + 125 µg NFIF in 4 ml H_2O*	4 ml H_2O*	68
20,000 pg B_{12} + 125 µg NFIF in 4 ml H_2O*	4 ml H_2O*	36
40,000 pg B_{12} + 125 µg NFIF in 4 ml H_2O*	4 ml H_2O*	18

* Deionized water.

It was further observed (Figure 1) that several gastric juices from patients with pernicious anemia gave greater uptake of vitamin B_{12} at concentrations of 0.005 ml to 0.05 ml than at 0.1 ml. This "paradoxical curve" has been observed in 8 out of 20 specimens of gastric juice from pernicious anemia patients assayed in serial concentrations, including basal and histamine-stimulated specimens, and may suggest the presence of an intrinsic factor inhibitor that can be removed by dilution.

Gastric juice from one patient with pernicious anemia resulted in less B_{12} uptake than that of the saline control, perhaps owing to the presence of nonintrinsic factor B_{12} binders (or, alterna-

tively, owing to the presence of nonfunctional intrinsic factor binding B_{12}, or intrinsic factor inhibitors).

Determination of B_{12}-binding capacity. The B_{12}-binding capacity of 125 μg (0.05 ml) NFIF was determined by dialysis with Visking casing, as previously described (35). Of 5,000 pg B_{12} added (Table II), 88% was bound by 125 μg of NFIF, indicating a binding capacity of approximately 35 pg B_{12} per μg IF. This is 0.1 the binding capacity previously found (35) for Lederle preparation Wes 671-A (323 pg B_{12} per μg IF), and is an example of correlation of binding capacity and clinical potency, since NFIF is clinically 0.1 as potent (50 mg = 1 USP unit) as Wes 671-A (5 mg = 1 USP unit) (16). As will be demonstrated below, such a correlation does not uniformly occur (Table X) (36).

Effect of varying concentrations of $Co^{57}B_{12}$. Various amounts of $Co^{57}B_{12}$ from 78 to 5,000 pg were added to the standard assay system. Figure 2 shows that with concentrations of 1,250 to 5,000 pg per flask in the presence of intrinsic factor, the uptake of B_{12} by the homogenate is a

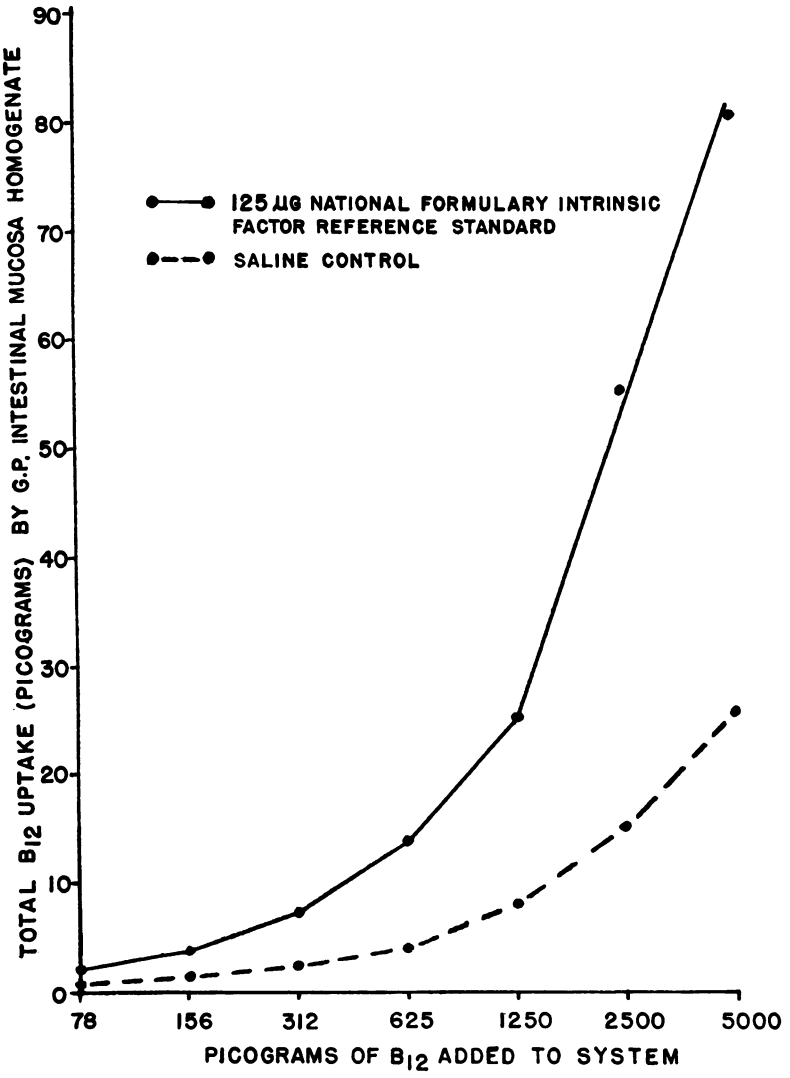

FIG. 2. EFFECT OF VARIOUS CONCENTRATIONS OF $Co^{57}B_{12}$, UP TO 5,000 PICOGRAMS, ON VITAMIN B_{12} UPTAKE BY GUINEA-PIG INTESTINAL MUCOSA HOMOGENATE. Note that abscissa scale is not arithmetic.

TABLE III

Effect of glucose, hyperoxia, anoxia, and 2,4-dinitrophenol on standard guinea-pig intestinal mucosa homogenate assay

Incubation medium	Specimen	B_{12} Uptake by homogenate
		pg
Experiment A		
Standard	Saline control	78
Standard	NFIF	154
Standard + 200 mg/100 ml glucose	NFIF	156
Experiment B		
Standard; gaseous phase—air	Saline control	57
Standard; gaseous phase—air	NFIF	192
Standard; gaseous phase—95 % O_2–5 % CO_2	NFIF	202
Standard; gaseous phase—100 % N_2	NFIF	197
Experiment C		
Standard	Saline control	21
Standard	NFIF	112
Standard + 2, 4-DNϕ*	NFIF	102
Standard	NHGJ†	97
Standard + 2,4-DNϕ	NHGJ	93

* 2,4-DNϕ = 2,4-dinitrophenol, 10^{-4}M.
† NHGJ = Normal human gastric juice, 0.1 ml.

direct function of the amount of B_{12} added. The ratio of the intrinsic factor curve to the control curve is approximately the same (3.1 to 4.0) at these concentrations. With higher B_{12} concentrations (7,500 and 10,000 pg per flask), the curve ceased to be linear (Figure 3). Because 5,000 pg gave the largest B_{12} uptake without loss of linearity, this amount was chosen for the assay system.

Effect of glucose, hyperoxia, anoxia, and 2,4-dinitrophenol. Table III summarizes three experiments suggesting that glucose (200 mg per 100 ml), hyperoxia, anoxia, and 2,4-dinitrophenol (10^{-4} M) do not affect B_{12} uptake by the assay system.

Rate of uptake of B_{12} by homogenate. To determine the rate of uptake of B_{12} by the homogenate, two series of duplicate tubes containing 0.1 ml and 0.2 ml normal human gastric juice and a third series of saline controls were incubated at room temperature for periods of from 5 minutes to 4 hours. At the end of the incubation periods, the homogenate was immediately precipitated at 2,000 × *g* in the cold and washed twice, and its radioactivity was determined (Figure 4). The

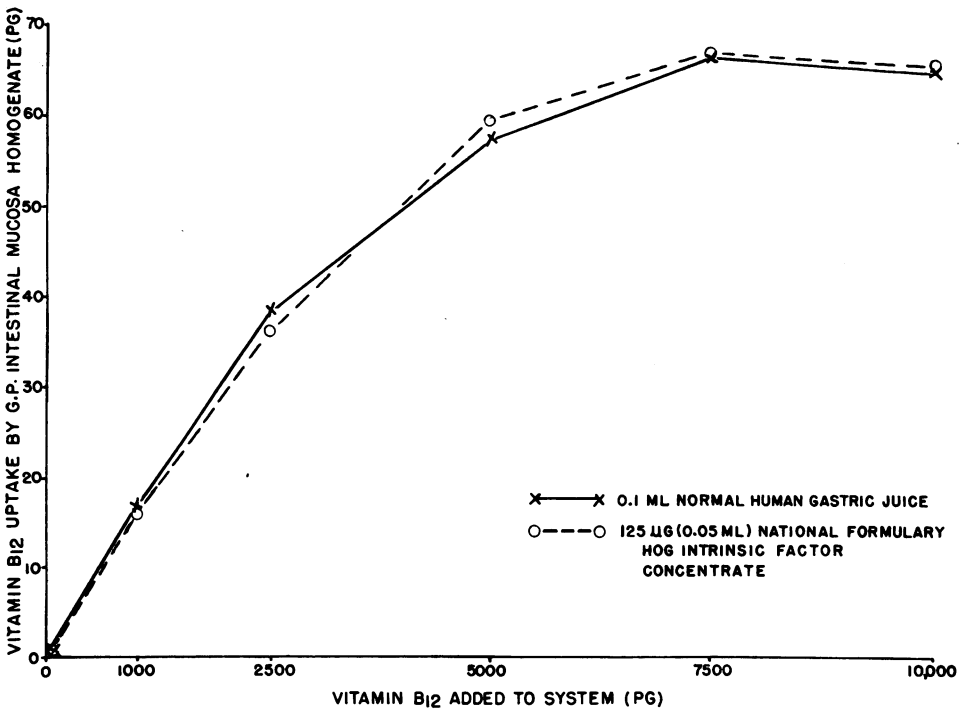

FIG. 3. EFFECT OF VARYING CONCENTRATIONS OF $Co^{57}B_{12}$, UP TO 10,000 PICOGRAMS, ON VITAMIN B_{12} UPTAKE BY GUINEA-PIG INTESTINAL MUCOSA HOMOGENATE. Abscissa scale is arithmetic.

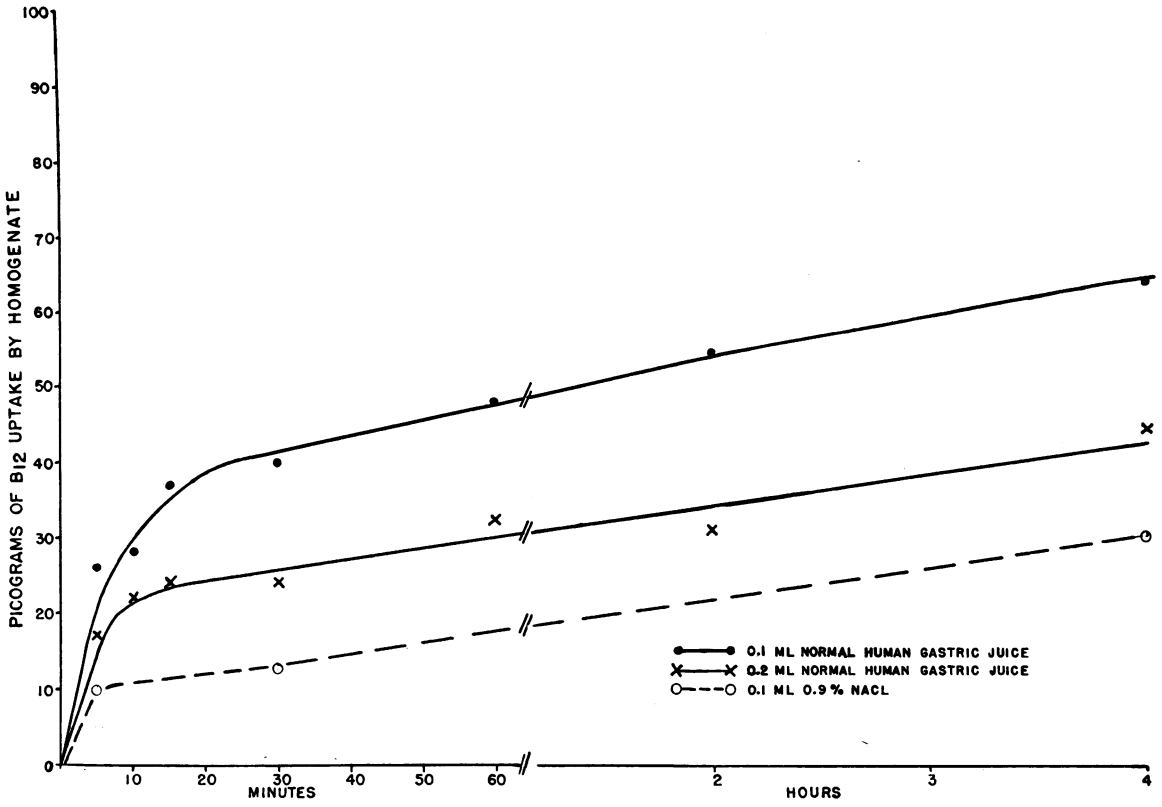

FIG. 4. RATE OF UPTAKE OF VITAMIN B_{12} IN STANDARD GUINEA-PIG INTESTINAL MUCOSA HOMOGENATE ASSAY.

rate of B_{12} uptake was noted to be most rapid in the first 15 minutes and resembled a first-order reaction. From 30 minutes to 4 hours, the uptake was linear, at a much slower rate, and judged by the control rise, possibly not dependent upon intrinsic factor. The curve for 0.2 ml gastric juice was parallel to the curve for 0.1 ml, but the quantitative B_{12} uptake was less, suggesting that excess intrinsic factor was present, consistent with the results of earlier experiments summarized in

TABLE IV

Effect of temperature on standard guinea-pig intestinal mucosa homogenate assay

Temperature	Sample	B_{12} Uptake by homogenate	$\frac{B_{12}\text{ Uptake of sample}}{B_{12}\text{ uptake of control}}$
		pg	
Experiment D			
3° C	Saline control	11	1.0
3° C	NHGJ	137	12.8
3° C	NFIF	66	6.1
25° C	Saline control	18	1.0
25° C	NHGJ	168	9.5
25° C	NFIF	135	7.6
Experiment E			
25° C	Saline control	14	1.0
25° C	NHGJ	88	6.3
25° C	NFIF	85	5.1
37° C	Saline control	20	1.0
37° C	NHGJ	139	6.8
37° C	NFIF	114	5.6

TABLE V

Effect of pH on standard guinea-pig intestinal mucosa homogenate assay

Buffered medium, pH	B_{12} Uptake by homogenate		
	Saline	NHGJ	NFIF
	pg	*pg*	*pg*
Experiment F			
5.4	4.9	4.9	2.0
5.8	4.6	4.6	3.0
6.2	4.5	15.4	16.9
6.6	4.4	38.0	42.0
7.2	6.0	36.6	40.0
7.7	7.5	37.4	39.2

Figure 1. The B_{12} uptake by the saline control flask also increased with time, so that by the end of 4 hours, the ratio of sample uptake to control uptake was less than at 30 minutes (2.1 vs. 2.5). For these reasons, 30 minutes was chosen as the usual incubation time.

Effect of temperature on uptake of B_{12} by homogenate. Two experiments to determine the effect of various temperatures on uptake of B_{12} are summarized in Table IV. As the temperature was increased, the uptake of B_{12} was increased in all samples, including the saline control. As a result, there was no consistent or significant change in the B_{12} uptake of sample: B_{12} uptake of control ratio with increasing temperature.

pH dependence of system. At pH 5.4 to 5.8, there is no enhancement of B_{12} uptake by intrinsic factor; enhancement is evident at pH 6.2, and is much greater at pH 6.6 to 7.7 (Table V).

Effect of incubation of gastric juice at pH 1.5 before assay. Incubation of normal human gastric juice (with or without intact peptic activity) at pH 1.5 and 37° C for 18 hours before assay destroys its ability to enhance B_{12} uptake by the homogenate (experiment G in Table VI). A sample of the same gastric juice neutralized to pH 7.0, not previously incubated at 37° C, enhanced B_{12} uptake seven times.

Effect of exposure to alkali. Normal human gastric juice was brought at room temperature to pH 11 for 20 minutes to destroy its peptic activity. Thereafter, the specimen was back-titrated to pH 7. The activity of the specimen was then compared to the activity of a sample of the same gastric juice that had been directly titrated to pH 7 immediately after collection. As shown in experiment H, no difference in the ability of the specimens to enhance B_{12} uptake by the homogenate was demonstrated.

Effect of heating gastric juice. Experiment J (Table VII) shows that heating normal human gastric juice to 100° C for 5 minutes almost completely destroys its activity in the system. The hog intrinsic factor reference standard was similarly affected, to a lesser extent.

Effect of heating intestinal homogenate. Heating the intestinal homogenate to 100° C for 3 minutes destroys its intrinsic factor receptors, as shown by experiment J. In the presence of normal human gastric juice or hog intrinsic factor concentrate, the radioactive B_{12} uptake by the

TABLE VI

Effect of prior incubation of normal human gastric juice at pH 1.5 and pH 11 on standard guinea-pig intestinal mucosa homogenate assay

Sample	B_{12} Uptake by homogenate	B_{12} Uptake of sample / B_{12} uptake of control
	pg	
Experiment G		
NHGJ,* no. 27	112	7.2
NHGJ,† no. 27, acid-incubated	17	1.1
Saline control	16	1.0
Experiment H		
NHGJ,* no. 22	104	2.6
NHGJ,‡ no. 22, alkali-incubated	104	2.6
Saline control	40	1.0

* NHGJ—0.1 ml normal human gastric juice neutralized immediately after collection.

† Acid-incubated NHGJ—0.1 ml normal human gastric juice, incubated at pH 1.5 for 18 hours immediately after collection and before neutralization.

‡ Alkali-incubated NHGJ—normal human gastric juice titrated to pH 11 immediately after collection for 20 minutes, then neutralized.

heated homogenate is even lower than that of the saline control. This probably is the result of B_{12} binding by intrinsic factor, preventing passive diffusion of B_{12} into the homogenate. Heated human gastric juice or hog intrinsic factor concentrate gives values equal to those of the saline control.

Efficacy of acetone-washed lyophilized homogenate. Experiment K in Table VIII shows the comparative activities of lyophilized homogenate and of fresh homogenate. For comparison purposes, a single batch of homogenate was prepared, half of it being subsequently lyophilized. Equivalent amounts of fresh and lyophilized homogenate were run in separate flasks in an assay of a specimen of normal human gastric juice and the hog intrinsic factor reference standard. The results indicate that lyophilization does not destroy intrinsic factor receptors in the homogenate and that results are similar with either fresh or lyophilized material. The lyophilized material has retained sufficient activity after being stored at room temperature for 6 months to be useful in the assay system (experiment L).

Effect of storing fresh intestinal homogenate at − 20° C. Fresh homogenate stored at − 20° C for 8½ months has intact intrinsic factor receptors as demonstrated in experiment M.

Efficacy of lyophilized mixture of homogenate, KRT buffer, and $Co^{57}B_{12}$. Attempts to prepare a consistently reliable "dry mix" of homogenate, buffer, and radioactive B_{12} in a single lyophilized

TABLE VII

Effect of prior heating on intrinsic factor and on intestinal homogenate in standard assay

Sample	B_{12} Uptake by homogenate	B_{12} Uptake of sample / B_{12} uptake of control
	pg	
Experiment J		
Unheated homogenate		
NHGJ	112	5.5
NHGJ, heated*	26	1.3
NFIF	132	6.5
NFIF, heated*	55	2.7
Saline control	20	1.0
Heated homogenate*		
NHGJ	8	0.6
NHGJ, heated*	15	1.2
NFIF	7	0.6
NFIF, heated*	11	0.9
Saline control	12	1.0

* Heated to 100° C for 5 minutes before assay.

TABLE VIII

Efficacy of various fresh and lyophilized guinea-pig intestinal homogenate preparations

Sample	Fresh homogenate		Lyophilized homogenate	
	B_{12} Uptake	B_{12} Uptake of sample / B_{12} uptake of control	B_{12} Uptake	B_{12} Uptake of sample / B_{12} uptake of control
	pg		*pg*	
Experiment K: Comparative activities of fresh and lyophilized homogenates				
NHGJ, no. 53	93	6.2	119	10.2
NFIF	108	7.2	83	7.2
Saline control	15	1.0	12	1.0
Experiment L: Activity of lyophilized homogenate stored at room temperature for 6 months				
NHGJ, no. 53			81	3.9
NHGJ, no. 60			66	3.2
Saline control			21	1.0
Experiment M: Activity of fresh intestinal homogenate stored for 8½ months at −20° C				
NHGJ, no. 53	95	5.1		
NFIF	65	3.5		
Saline control	19	1.0		
Experiment N: Activity of lyophilized mixture of intestinal homogenate, Krebs-Ringer–Tris buffer and $Co^{57}B_{12}$ as an assay substrate for intrinsic factor				
NHGJ, no. 53			34	2.6
PAGJ*			7	0.5
NFIF			19	1.4
Saline control			13	1.0

* PAGJ = gastric juice from a patient with pernicious anemia.

TABLE IX

Effect of intrinsic factor antibodies on standard guinea-pig intestinal mucosa homogenate assay

Sample	B_{12} Uptake by homogenate	B_{12} Uptake of sample / B_{12} uptake of control
	pg	
Experiment O		
NHGJ	58	5.4
NHGJ + normal rabbit serum*	62	5.6
NHGJ + rabbit antihuman intrinsic factor†	15	1.3
Saline control	11	1.0
Experiment P		
NFIF	60	4.0
NFIF + rabbit antihuman intrinsic factor‡	12	0.8
NHGJ	26	1.7
NHGJ + rabbit antihog intrinsic factor§	11	0.7
Saline control	15	1.0

* Normal rabbit serum, 0.2 ml.
† Rabbit antihuman stomach mucosa serum, 0.2 ml.
‡ Rabbit antihuman stomach mucosa serum, 0.1 ml.
§ Rabbit antihog serum, 0.1 ml.

substance have been only partially successful. This may be due to osmotic damage to the intrinsic factor receptors occurring during lyophilization with KRT buffer. Experiment N represents an assay in which this "dry mix" was used. Although there is enhancement of B_{12} uptake by the "dry-mix" homogenate with normal gastric juice and hog intrinsic factor concentrate, the uptake was only $\frac{1}{5}$ to $\frac{1}{3}$ of that obtained with fresh or lyophilized homogenate (experiment K)

TABLE X

Correlation of standard guinea-pig intestinal mucosa homogenate assay with modified Schilling test; lack of correlation between intrinsic factor activity and B_{12}-binding capacity of human gastric juice

Human gastric juice	Schilling test: % dose excreted	Homogenate assay: B_{12} Uptake of sample / B_{12} uptake of control	Percentage of 5,000 pg B_{12} bound by 0.1 ml gastric juice*
30 ml			
Normal			
20	4.5	2.7	11.4
24	10.9	2.8	41.2
21	7.7	3.3	17.7
25	6.0	3.5	
22	5.1	3.6	
23	7.6	3.6	15.4
Nonpernicious anemia			
50†	6.7	2.3	
63‡	16.9	3.9	
53§	22.8	6.8	16.9
Pernicious anemia			
39	1.5	1.1	
48	0.9	1.5	
66	1.5	1.2	41.4
35a‖	0.9	0.3	
35b‖	1.3	0.8	75.4
31	4.2	3.5	

* Binding capacity determined by dialysis as indicated in reference 35.
† Combined B_{12} and folic acid deficiency, 4 weeks postpartum.
‡ Folic acid deficiency.
§ Dietary B_{12} deficiency.
‖ Specimens from the same patient, 12 days apart.

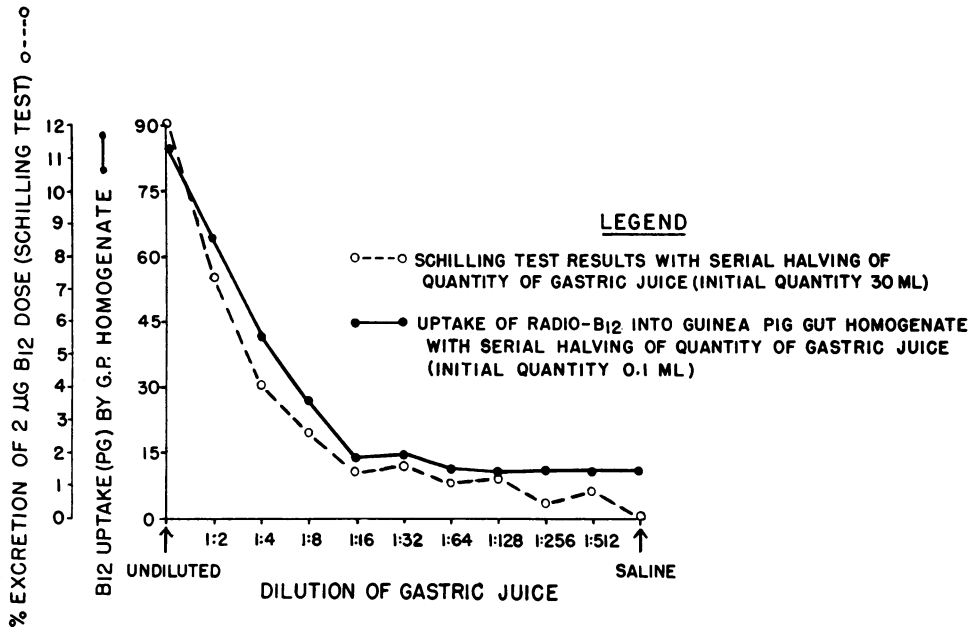

FIG. 5. CORRELATION OF *in vitro* STANDARD GUINEA-PIG INTESTINAL MUCOSA HOMOGENATE ASSAY WITH *in vivo* ASSAY (SCHILLING TEST) FOR INTRINSIC FACTOR ACTIVITY IN SERIAL DILUTIONS OF NORMAL HUMAN GASTRIC JUICE.

using the same gastric juice and hog intrinsic factor concentrate. However, our studies suggest that adding lyophilized KRT and radioactive B_{12} to lyophilized homogenate may result in a "dry mix" without significant loss of receptors.

Effect of intrinsic factor antisera on in vitro assay. Potent rabbit antisera to hog intrinsic factor concentrate (37) and to human stomach mucosa [3] inhibited the B_{12} uptake mediated by hog intrinsic factor concentrate and by human gastric juice (Table IX). Cross-reactivity with heterologous antigen was exhibited by both antisera (Table IX).

Correlation of assay with clinical activity of gastric juices. To test the assay system for physiologic relevance, gastric juices from normal individuals and from patients with megaloblastic anemias of various etiologies were collected and assayed. This *in vitro* activity was then compared with *in vivo* activity, i.e., the ability of 30 ml of the unknown gastric juice to promote absorption of a 2-μg dose of $Co^{60}B_{12}$ in a Schilling-type test (31) performed on a group of patients with pernicious anemia. Table X demonstrates that all specimens from normal individuals and from patients with megaloblastic anemia owing to dietary deficiencies of B_{12} or folic acid produced a two-fold or greater enhancement of B_{12} uptake by the homogenate. These gastric juices gave normal Schilling test results when fed to pernicious anemia patients, indicating normal intrinsic factor activity.

Of six gastric juice specimens from patients with documented pernicious anemia, five showed insignificant *in vitro* and *in vivo* activity (Table X). The sixth pernicious anemia patient is of interest in that his gastric juice produced an enhancement of B_{12} uptake by the homogenate of $3\frac{1}{2}$ times. With 4 hours of continuous nasogastric suction, a total of 25 ml of viscous gastric juice with a pH of 8.0 was obtained. When fed with 2 μg of $Co^{60}B_{12}$ to another patient with pernicious anemia, 4.2% of the administered radioactivity was excreted in the urine, suggesting the presence of significant amounts of intrinsic factor, as indicated by the *in vitro* assay.

No correlation was found between the B_{12}-binding activity of gastric juice, as determined by dialysis, and the *in vivo* or *in vitro* intrinsic factor activity of gastric juice (Table X). Indeed, the gastric juice which had the greatest B_{12}-binding

[3] Prepared and supplied by Dr. Manuel Kaplan.

capacity was from a patient with pernicious anemia and was without activity in the *in vivo* or *in vitro* assays.

The correlation between the *in vivo* and *in vitro* assays was studied further by making serial dilutions of a sample of normal human gastric juice with 0.9% saline. Thirty-ml samples of each dilution of gastric juice were serially fed with 2 μg of $Co^{60}B_{12}$ in Schilling-type tests (31) to a single patient with pernicious anemia in remission. The intrinsic factor activity of the dilutions of gastric juice was compared with the ability of 0.1 ml of each dilution to enhance radioactive B_{12} uptake in the standard guinea-pig gut homogenate assay. The results are plotted in Figure 5. In both *in vivo* and *in vitro* assays, the gastric juice showed parallel intrinsic factor activity. In 1:16 dilution and beyond, there was no significant intrinsic factor activity demonstrable by either assay, the *in vivo* assay resulting in less than 2% excretion of the radioactive B_{12} dose, and the *in vitro* assay showing uptake of radioactive B_{12} no greater than that of the saline control.

Effect of histamine on intrinsic factor secretion. Comparisons of intrinsic factor activity of gastric juice obtained under basal conditions with that of gastric juice obtained after maximal histamine stimulation are shown in Table XI. After histamine stimulation, in three of five normal subjects, the intrinsic factor activity per unit volume of gastric juice increased by factors of 1.6, 4.7, and 23.5, respectively, and there was also a two- to fivefold increase in volume of gastric secretion during the 45-minute collection periods. In Subject 22, the volume of secretion obtained after histamine stimulation was less than the basal volume, but the total secretion of intrinsic factor was unchanged because of the greater intrinsic factor activity per 0.1 ml of the posthistamine gastric

TABLE XI

Comparison of in vitro intrinsic factor activities of human gastric juice obtained before and after maximal histamine stimulation

Source of gastric juice	Gastric juice secreted in 45 minutes		Amount of B_{12} uptake facilitated by 0.1 ml gastric juice*		Ratio of activity of gastric juice	After histamine / before histamine
	Before histamine	After histamine	Before histamine	After histamine		
	ml	*ml*	*pg*	*pg*	*/0.1 ml*	*/45-min sample*
Normal subjects						
20	53	123	29	48	1.6	3.7
21	40	203	15	70	4.7	24.0
22	106	68	52	78	1.5	1.0
23	83	185	51	32	0.6	1.3
24	26	132	2	47	23.5	120.0
Folic acid-deficient patients						
60	22	98	65	63	1.0	4.5
63	22	79	81	0†		
Dietary B_{12}-deficient patient						
53	90	285	40	56	1.4	4.4
Pernicious anemia patients						
28	3	2	−21	−6	0	0
35	21	45	−7	−17	0	0
40	30	13	−3	1	1	0.4
41	4	6	−5	0	0	0
59	4	2	3	3	1	0.5

* Values represent the picograms of B_{12} taken up in excess of the uptake by the saline controls. A minus value means the amount of B_{12} uptake was less than that of the saline control.

† Histamine specimen moderately contaminated with blood.

juice. Subject 23 had a lower intrinsic factor activity per 0.1 ml gastric juice in the posthistamine specimen. Because of the greater volume of gastric juice, however, intrinsic factor secretion was greater in the posthistamine period. In one patient (no. 63) with folic acid deficiency, the posthistamine specimen contained a moderate amount of blood. This specimen was inactive in the *in vitro* system, possibly owing to blocking of intrinsic factor activity by blood group substance (38). None of the specimens from the patients with pernicious anemia showed significant intrinsic factor activity in the basal or posthistamine secretions, and several gave values less than the saline controls (indicated by the minus signs), suggesting the presence of nonintrinsic factor B_{12} binders.

DISCUSSION

The general acceptance of any *in vitro* assay depends on the demonstration of its ability to give results consistent with physiologic fact. Additional desirable characteristics are simplicity and reproducibility. The present studies suggest that a guinea-pig intestinal mucosa homogenate assay for human (and hog) intrinsic factor meets these requirements. This system has reliably shown the presence or absence of intrinsic factor in all specimens of gastric juice tested (including those from patients with free acid), as confirmed by the ability of the gastric juice to promote absorption of radioactive B_{12} in patients with pernicious anemia. All normal gastric juices gave a twofold or greater enhancement of vitamin B_{12} uptake by the homogenate.

The finding of detectable intrinsic factor activity in the gastric juice of one patient with pernicious anemia confirms the studies of three decades ago (2, 39) indicating the presence of intrinsic factor in the gastric juice of some pernicious anemia patients. Inadequate intrinsic factor output per day can thus result from secretion of an inadequate volume of gastric juice containing a normal concentration of intrinsic factor, from secretion of a normal volume of gastric juice containing a reduced concentration of intrinsic factor, or from appropriate combinations of these circumstances. *In vitro* assays for human intrinsic factor activity will simplify long-term studies (40, 41) delineating the natural history of the decline in intrinsic factor as pernicious anemia develops.

It should be noted that both *in vitro* and *in vivo* assays measure effective intrinsic factor activity and not necessarily total intrinsic factor content. The quantity of nonintrinsic factor B_{12} binders (and other inhibitors) may vary from one gastric juice to another. Both assays measure the net physiologic result of an unknown number of units of intrinsic factor plus an unknown number of inhibitors. The observation that at concentrations of 0.005 to 0.01 ml of gastric juice from some pernicious anemia patients, there was slight enhancement of B_{12} uptake, which was not demonstrable at higher concentrations, suggests the possibility that an "inhibitor" of intrinsic factor may have been diluted out at the lower concentrations.

Since intrinsic factor has not yet been isolated in pure form, any *in vitro* assay may be suspect. Supporting the validity of the methodology here described as an assay for human intrinsic factor activity are the following observations: *a*) the material in human gastric juice which enhances radioactive B_{12} uptake by guinea pig intestinal mucosa homogenate resembled human intrinsic factor in its destruction by heat (2) or prolonged exposure to acid before or after inactivation of pepsin (3), and in its resistance to moderate alkalinity (4) for a short period or storage for weeks at $-20°$ C at neutral pH (1); *b*) intestinal homogenate will not serve as a source of "receptors" after heating or at pH 5.8 or lower (5–7), suggesting that the system is not measuring a nonspecific binding effect; *c*) 4 samples of colostrum and 8 samples of saliva, substances known to have B_{12}-binding properties, do not enhance B_{12} uptake by the homogenate, and there was no relation between B_{12}-binding power and assay potency of the various gastric juices; *d*) immunologic studies show that a potent rabbit antiserum to human stomach mucosa blocks the activity of human gastric juice and hog intrinsic factor concentrate in the homogenate system and that rabbit antihog intrinsic factor antibody inhibits the action of human gastric juice and hog intrinsic factor concentrate.

The assay system was not affected by anoxia, hyperoxia, glucose, or 2,4-dinitrophenol. With 5,000 pg radioactive B_{12}, increasing concentrations of normal gastric juice up to 0.1 ml resulted

in increased radioactive B_{12} uptake by the homogenate. Beyond 0.1 ml, there was no significantly greater enhancement; with much larger quantities there was some inhibition of B_{12} uptake. This phenomenon suggests that with 0.1 ml the "receptors" on the intestinal homogenate are nearly saturated (16, 17). The rate of uptake of radioactive B_{12} by the homogenate was rapid during the first 15 to 30 minutes, but a continued, slower uptake was observed for as long as 4 hours, paralleled, however, by the saline control.

Guinea-pig intestinal mucosa homogenate "receptors" for intrinsic factor appear to remain essentially intact after lyophilization, acetone washing, and relyophilization. This makes possible the preparation of large quantities of lyophilized material to serve as a substrate that may be stored at room temperature and used as needed for repeated assays of human intrinsic factor. Vitamin B_{12} binding cannot be used as a measure of intrinsic factor activity in unfractionated human gastric juice. Of the gastric juices in which B_{12}-binding capacity was measured, the one with greatest binding capacity had no *in vivo* or *in vitro* intrinsic factor activity. Many others have previously noted the lack of correlation between total B_{12}-binding capacity of gastric juice and intrinsic factor activity (36, 42, 43) and the fact that many materials other than intrinsic factor bind vitamin B_{12} (43).

The *in vitro* guinea-pig intestinal mucosa homogenate assay technique can be incorporated into routine gastric analysis, and results are available the same day. It requires no administration of radioactivity to the patient and no urine or stool collections or body surface scanning. It appears to be a more direct measure of intrinsic factor activity than *in vivo* assays measuring fecal, serum, liver, urine, or whole body radioactivity, since it is performed on the gastric juice itself.

Finally, it must again be emphasized that too much intrinsic factor will result in lowered vitamin B_{12} uptake by the homogenate because of the critical nature of the ratios vitamin B_{12} : intrinsic factor : receptors (7). The importance of this point is illustrated by the fact that others were unsuccessful (44) in attempts to confirm our initial indication (7) that guinea-pig gut homogenate could be used for intrinsic factor assay, until our suggestion (45) to use 0.02 ml of human gastric juice was adopted (46). Subsequently, we reported (26) use of the ratio 0.1 ml gastric juice : 5,000 pg B_{12} and, using that ratio, others confirmed that report in detail (47).

The finding that the intrinsic factor activity of gastric juice from six of eight subjects without pernicious anemia was greater in the posthistamine specimens suggests that, in addition to the enhancement of hydrochloric acid secretion and pepsin secretion (48–51), histamine stimulates intrinsic factor secretion. Since present evidence suggests that both pepsin (51) and intrinsic factor (52) are products of the chief cell, the increase in intrinsic factor secretion after histamine is not surprising. The possibility that the increased intrinsic factor activity represents a "washing-out" of intrinsic factor from the glandular crypts cannot at present be excluded.

SUMMARY

Homogenate of mucosa from the distal half of the guinea-pig small intestine appears to provide a simple, reliable, rapid *in vitro* assay for intrinsic factor, which correlates well with *in vivo* assays. The present study demonstrates this with both human and hog intrinsic factor.

The specificity of the system for intrinsic factor is further suggested by the findings that the activity of gastric juice in the homogenate system, like the activity of intrinsic factor, is reversibly inhibited at pH 5.8 and below, and is destroyed by heat or prolonged exposure to acid, but not by moderately alkaline pH or by storage at − 20° C at neutral pH. Antibody to human stomach mucosa inhibits the uptake of B_{12} mediated by human gastric juice. B_{12}-binding proteins other than intrinsic factor do not enhance B_{12} uptake by the homogenate.

The homogenate may be stored frozen, or lyophilized and stored at room temperature for use as needed. The assay may be incorporated into routine gastric analysis, requires less than 1.0 ml of gastric juice, is a direct measure of intrinsic factor activity, and can provide results within an hour.

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ADDENDUM

Lyophilized homogenate has been shown to retain normal activity after storage for 16 months at room temperature. In further immunologic studies, we demonstrated that rabbit antihog intrinsic factor antibody completely destroys the activity of NFIF in the homogenate system. Maintenance of gastric juice at pH 7 for 18 hours at 37° C did not significantly reduce its activity when pepsin had been previously inactivated.

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