JCI The Journal of Clinical Investigation

# Identification of a macromolecular factor in the ileum which binds intrinsic factor and immunologic identification of intrinsic factor in ileal extracts

Sheldon P. Rothenberg

*J Clin Invest.* 1968;47(4):913-923. https://doi.org/10.1172/JCI105783.

Research Article

The precipitate which resulted when $^{57}CoB_{12}$ bound to normal human gastric juice was subjected to a 15% concentration of $Na_2SO_4$ contained virtually no radioactivity. However, after in vivo incubation of the gastric juice-$^{57}CoB_{12}$ mixture in the distal ileum of the guinea pig, the dialyzed extract of the washed mucosa contained a fraction of $^{57}CoB_{12}$ which was precipitated at 15% $Na_2SO_4$. In addition, in vitro incubation of gastric juice-$^{57}CoB_{12}$ with an extract of the ileal mucosa or brush border membranes also resulted in the formation of a 15% $Na_2SO_4$-insoluble fraction which contained $^{57}CoB_{12}$. The formation of this $^{57}CoB_{12}$-containing insoluble fraction did not occur or was diminished by (*a*) addition of an excess of $B_{12}$-free normal human gastric juice. (*b*) reducing the incubation pH to 2, (*c*) incubating the mixture at 4°C, (*d*) pretreating the ileal extract at 56°C for 30 min, (*e*) incubating the reaction in sodium EDTA but not calcium EDTA, (*f*) incubating gastric juice-$^{57}CoB_{12}$ with an extract of jejunal mucosa. Sephadex gel filtration was used to demonstrate that the factor in the ileal extract which reacted with the gastric juice-$^{57}CoB_{12}$ filtered through G-100 and G-200 columns in the excluded volume.

When the ileal extract obtained after in vivo incubation with gastric juice-$^{57}CoB_{12}$ was subjected to starch gel electrophoresis one peak of radioactivity remained at the origin and another moved […]

**Find the latest version:**

https://jci.me/105783/pdf

# Identification of a Macromolecular Factor in the Ileum Which Binds Intrinsic Factor and Immunologic Identification of Intrinsic Factor in Ileal Extracts

Sheldon P. Rothenberg with the technical assistance of Anna Liisa Huhti

*From the Blood Research Laboratory, Department of Medicine, New York Medical College, New York 10029*

Abstract The precipitate which resulted when $^{57}CoB_{12}$ bound to normal human gastric juice was subjected to a 15% concentration of $Na_2SO_4$ contained virtually no radioactivity. However, after in vivo incubation of the gastric juice–$^{57}CoB_{12}$ mixture in the distal ileum of the guinea pig, the dialyzed extract of the washed mucosa contained a fraction of $^{57}CoB_{12}$ which was precipitated at 15% $Na_2SO_4$. In addition, in vitro incubation of gastric juice–$^{57}CoB_{12}$ with an extract of the ileal mucosa or brush border membranes also resulted in the formation of a 15% $Na_2SO_4$-insoluble fraction which contained $^{57}CoB_{12}$. The formation of this $^{57}CoB_{12}$-containing insoluble fraction did not occur or was diminished by (*a*) addition of an excess of $B_{12}$-free normal human gastric juice, (*b*) reducing the incubation pH to 2, (*c*) incubating the mixture at 4°C, (*d*) pretreating the ileal extract at 56°C for 30 min, (*e*) incubating the reaction in sodium EDTA but not calcium EDTA, (*f*) incubating gastric juice-$^{57}CoB_{12}$ with an extract of jejunal mucosa. Sephadex gel filtration was used to demonstrate that the factor in the ileal extract which reacted with the gastric juice-$^{57}CoB_{12}$ filtered through G-100 and G-200 columns in the excluded volume.

When the ileal extract obtained after in vivo incubation with gastric juice-$^{57}CoB_{12}$ was subjected to starch gel electrophoresis one peak of radioactivity remained at the origin and another moved anodally. Eluates of each peak reacted with anti-intrinsic factor antibody indicating that at least the immunologically reacting portion of the intrinsic factor molecule was present in two fractions with different electrophoretic mobility.

These studies indicate that immunologically intact intrinsic factor can be extracted from the ileum after in vivo incubation with gastric juice-$^{57}CoB_{12}$, and that a macromolecular factor is present in the distal ileal mucosa which binds intrinsic factor both in vitro and in vivo, changing its solubility and electrophoretic properties. It is suggested that this ileal binding factor is the previously postulated intestinal receptor for intrinsic factor.

## INTRODUCTION

The intestinal absorption of vitamin $B_{12}$ is dependent on a complex series of events requiring, as Castle originally discovered (1), the presence of a heat-labile factor in gastric juice, which has been designated intrinsic factor (IF). It has been established that IF binds (2) and then carries the $B_{12}$ molecule to the ileum where, in man (3) and other animals (4, 5) absorption of the vitamin takes place.

More enigmatic is the specific mechanism which facilitates the passage of the large, water-soluble $B_{12}$ molecule (mol wt 1365) through an intact mucosal cell. Indeed, the mystery of this absorptive process is compounded by the firm attachment of the vitamin to IF, a large carrier protein. It has been shown in man (6) and rat (4, 7) that the absorption of $B_{12}$ is a time consuming process

Address requests for reprints to Dr. Sheldon Rothenberg, New York Medical College, 1249 Fifth Avenue, New York 10029.

which is probably not energy dependent, for metabolic inhibitors have either no (8) or only a slight inhibitory effect (9).

The role of IF in facilitating the absorption of $B_{12}$ at the cellular level once it has transported the vitamin through the lumen of the small intestine has been the subject of many investigations. Although Strauss, Wilson, and Hotchkiss (5) reported that there was no apparent binding of free IF or free $B_{12}$ to intestinal epithelial cells, there is other evidence, direct and indirect, that the IF-$B_{12}$ complex and probably free IF do in fact attach to the intestinal mucosa. Nieweg, Shen, and Castle in 1957 (10) showed that rat gastric juice enhanced $B_{12}$ absorption in the rat when given simultaneously with $B_{12}$, but not when it preceded the administration of $B_{12}$. They therefore suggested that a site on the intestinal mucosa was occupied by the free IF thereby inhibiting the uptake of the vitamin. Abels and coworkers (11) also demonstrated that preincubation of rat intestine with rat gastric juice inhibited absorption of $B_{12}$ and postulated the presence of an IF-receptor protein which facilitated the entry of IF-$B_{12}$ into the cell. Herbert (12) using everted sacs of rat small intestine demonstrated that excess hog IF, calcium-free solution, and EDTA inhibited the uptake of IF-bound $B_{12}$ and suggested that there was an intestinal receptor site for IF. Cooper and Castle (8), using the perfused rat ileum, also showed that EDTA inhibited IF-mediated $B_{12}$ uptake when present in the perfusate but did not decrease intestinal uptake when used as a rinsing solution following the perfusion. They concluded, therefore, that adsorption of the IF-$B_{12}$ complex to the intestinal mucosa was a phase of $B_{12}$ absorption.

More direct identification of IF on or in the intestinal mucosa was made by Boass and Wilson (13) who demonstrated that the soluble supernate of hamster ileal sacs exposed to the IF-$B_{12}$ complex had IF activity when introduced into another hamster ileal sac. Cooper (14) has also reported that the washings of guinea pig intestinal sacs with EDTA or acetic acid after incubation with gastric juice have IF activity. More recently Ukyo and Cooper (15) demonstrated biological IF activity in the supernate and sediment of a saline extract of guinea pig intestine after administration of $^{57}CoB_{12}$ bound to normal human gastric juice.

This report will describe both the identification of immunologically intact IF in ileal extracts after in vivo incubation with $B_{12}$ bound to normal human gastric juice, and the identification, extraction, and properties of a factor in ileal extract which binds IF and which may be the hypothetical IF-receptor site of the intact ileum proposed by Herbert (12).

## METHODS

High specific activity $^{57}CoB_{12}$ (120–170 $\mu c/\mu g$) was used for all the studies to be described.[1] All new samples of $^{57}CoB_{12}$ were chromatographed with stable cyanocobalamin using water-butanol-ammonia (1%) as the solvent, and found to be 98% or more pure. Normal human gastric juice (NHGJ) obtained after augmented histamine stimulation (16) was the source of intrinsic factor (IF). The $B_{12}$-binding capacity of the gastric juice was determined by saturating it with $^{57}CoB_{12}$ and determining the total $^{57}CoB_{12}$ bound by precipitation of all proteins with $ZnSO_4$ and $Ba(OH)_2$ (17). The mixtures of NHGJ and $^{57}CoB_{12}$ were then made accordingly with the aim of having 90% of the radioactivity bound and thus avoiding any significant excess of free IF. The unit of weight preceding the terms NHGJ-$^{57}CoB_{12}$ or $^{57}CoB_{12}$ always refers to the content of $B_{12}$. Anti-IF antiserum was obtained from a patient with pernicious anemia found to contain the binding type of anti-IF antibody (18).

Most radioactivity was assayed in a 2 inch, well-type scintillation crystal with a gamma ray spectrometer and scaler. The radioactivity of tissue specimens was assayed in a 3 inch, well-type scintillation crystal.

### Guinea pig ileal extracts

Extracts of guinea pig ileal mucosa were obtained with and without prior incubation of the ileum with NHGJ-$^{57}CoB_{12}$. The in vivo incubations were carried out by anesthetizing a fasted guinea pig with ether and isolating with ligatures the terminal 15–17 cm of ileum. A mixture of 0.3 ml of NHGJ, 0.1 ml of a solution containing 50–80 ng/ml of $^{57}CoB_{12}$, and 3.6 ml of Ringer's-bicarbonate solution, pH 7.2, containing 0.217 g $NaHCO_3$ per 100 ml of Ringer's solution, was then introduced with a needle and syringe into this isolated ileal segment. After 1 hr the animal was killed and the isolated ileal segment removed, opened longitudinally, and washed with cold Ringer's-bicarbonate solution until there was no further decrease in radioactivity. The mucosa was then scraped off with a glass slide, and similarly washed until the radioactivity remained constant.

The washed mucosa was suspended in 4 ml of Ringer's-bicarbonate solution and homogenized for 30 sec in an ice-jacketed microunit of a Waring Blendor. The homoge-

[1] Purchased from Radiochemical Centre, Amersham, England.

nate was then mechanically disrupted with glass beads [2] (0.5 g/ml) for 3–5 min in a Braun mechanical fractionator cooled with liquid $CO_2$. This step effectively disrupted all parts of the mucosal cells including brush border membranes as determined by microscopic examination. The cellular fragments were separated by centrifugation for 30 min (30,000 *g*) at 4°C and the supernatant fractions dialyzed against Ringer's-bicarbonate at this temperature for 12–24 hr. For descriptive purposes in the text to follow, such extracts containing $^{57}CoB_{12}$ are termed labeled extracts. Ileal mucosa extracts obtained by the above method but without prior incubation with NHGJ-$^{57}CoB_{12}$ are termed unlabeled extracts.

Brush border extracts were prepared by isolating the brush border membranes by the method of Miller and Crane (19). After washing the isolated membranes twice with 0.005 M EDTA and twice with Ringer's-bicarbonate, the preparation from a single ileum was resuspended with 2 g of glass beads in 2.5 ml of Ringer's-bicarbonate, subjected to mechanical disruption for 3 min, and the cellular particulate was then separated by centrifugation at 4°C (30,000 *g*).

The objective of the experiments to be described next is to show that gastric juice–bound $^{57}CoB_{12}$ undergoes a change in solubility and electrophoretic properties after it is incubated either with intact or with extracts of ileal mucosa. The experiments also aim to demonstrate that such changes are due to an interaction between the IF-$B_{12}$ complex and a factor in the ileal mucosa.

The solubility of bound $^{57}CoB_{12}$ in labeled ileal extract at 15% $Na_2SO_4$ was studied by incubating an aliquot of the extract with 0.3 ml of normal serum in sufficient Ringer's-bicarbonate for a total volume of 2.3 ml. After 30 min the 15% $Na_2SO_4$-insoluble proteins were precipitated with an equal volume (2.3 ml) of 30% $Na_2SO_4$ (w/v), and the quantity of $^{57}CoB_{12}$ coprecipitated was determined by assaying the radioactivity of the supernate. The solubility of NHGJ-$^{57}CoB_{12}$ at 15% $Na_2SO_4$ prior to the in vivo incubation was similarly studied. The purpose of the normal serum in these experiments was to increase the total protein concentration of the incubation mixtures in order to facilitate the precipitation of proteins at this salt concentration. Since gamma globulin is also insoluble at 15% $Na_2SO_4$, the coprecipitation of $^{57}CoB_{12}$ after NHGJ-$^{57}CoB_{12}$ is incubated with anti-IF antiserum is due to the binding of the IF-$^{57}CoB_{12}$ complex by the immune globulin (18). To preclude the transfer of any free $^{57}CoB_{12}$ to serum binding proteins, the normal serum and anti-IF antiserum used for all experiments were saturated with 4 ng per ml of crystalline $B_{12}$ and the excess unbound $B_{12}$ removed with powdered albumin–coated charcoal (300 mg/ml) (20).

The in vitro interaction between NHGJ-$^{57}CoB_{12}$ and extracts of ileal mucosa, isolated brush border membranes, and jejunal mucosa was studied by incubating 25 pg of the gastric juice–bound $^{57}CoB_{12}$ with 0.5 ml of each extract in sufficient Ringer's-bicarbonate for a total of 2.0 ml. After 30 min 0.3 ml of normal serum, and an equal volume of 30% $Na_2SO_4$ (2.3 ml) was added and the percentage of $^{57}CoB_{12}$ precipitated was determined by assaying the radioactivity of the supernate.

By appropriately changing the conditions of the reaction mixture the effect of different hydrogen ion concentrations and temperature on the in vitro interaction between NHGJ-$^{57}CoB_{12}$ and the ileal extract was investigated. The effect of a chelating agent on this interaction was also studied by substituting either sodium or calcium EDTA for the Ringer's-bicarbonate in the reaction mixture.

Labeled extract was subjected to starch gel electrophoresis as described by Smithies (21), with and without prior incubation with anti-IF antiserum. For one experiment the gel was prepared in 0.023 M borate buffer, pH 8.9, and for a second experiment the gel was prepared in a mixture of 0.5 M Tris, 0.0186 M EDTA, and 0.074 M boric acid, pH 8.8. For both gel systems the chamber buffer was 0.3 M borate buffer, pH 8.2. The electrophoresis was carried out at 4°C for 16 hr at 220 volts. The gel was then cut into 0.5 cm sections and counted in test tubes in the well-type scintillation crystal. When the peaks of radioactivity were identified, they were eluted by freezing and thawing the gel followed by the application of gentle pressure with a spatula. The eluate from each of the two peaks was incubated separately with 0.3 ml of normal serum and 0.3 ml of anti-IF antiserum, and the fraction of radioactivity contained in the 15% $Na_2SO_4$-insoluble fraction was determined as described previously.

The filtration of unlabeled extract through Sephadex gel columns was also studied. Sephadex G-100 and G-200 were equilibrated with distilled water for 24 hr and columns of each, 20–25 cm in height, were prepared by gravity packing followed by washing with 0.9% NaCl. The void and excluded volumes were determined with dextran-blue, and the inner volumes by copper sulfate filtration. A 1 ml aliquot from a pool of six unlabeled ileal extracts was then applied to each of the two columns and filtered with 0.9% NaCl. The fractions constituting the excluded and inner volumes, respectively, were combined, dialyzed overnight at 4°C against distilled water, and then concentrated in the dialysis bag by fan evaporation to approximately 1 ml. A 0.5 ml aliquot of each concentrated fraction was incubated with 25 pg of NHGJ-$^{57}CoB_{12}$ in Ringer's-bicarbonate (total volume of 2.0 ml) for 30 min followed by the addition of 0.3 ml of normal serum and an equal volume of 30% $Na_2SO_4$. The $^{57}CoB_{12}$ remaining in the supernatant solution was then assayed.

## RESULTS

### Precipitation of free NHGJ-$^{57}CoB_{12}$, labeled ileal extract, and mixtures of unlabeled extracts and NHGJ-$^{57}CoB_{12}$ at 15% $Na_2SO_4$

The data summarized in Table I show that when NHGJ-$^{57}CoB_{12}$ was incubated with normal serum in Ringer's-bicarbonate and then subjected to a 15% concentration of $Na_2SO_4$, virtually no

[2] Size of glass beads ranged from 0.012 to 0.11 mm.

radioactivity coprecipitated with the insoluble proteins. However, after the NHGJ-$^{57}CoB_{12}$ was incubated in vivo in the guinea pig ileum, 56% of the bound $^{57}CoB_{12}$ in the mucosal extract then coprecipitated at this salt concentration, indicating that vitamin $B_{12}$ bound to NHGJ underwent some change in solubility property during incubation in the ileum. Most of the $^{57}CoB_{12}$ in the gastric juice used for the in vivo incubation was bound to IF since 69.5% of the radioactivity precipitated at 15% $Na_2SO_4$ after incubation with anti-IF antiserum.

To investigate whether a similar change in solubility characteristics of NHGJ-bound $^{57}CoB_{12}$ occurred in vitro, extracts of ileum, brush border membranes, and jejunum were incubated with NHGJ-$^{57}CoB_{12}$ and then subjected to a 15% concentration of $Na_2SO_4$ after the addition of normal serum as carrier protein. The data in Table II show that following such incubation with ileal extract, 48.1% of the radioactivity coprecipitated, while only 3.7% coprecipitated when the same NHGJ-$^{57}CoB_{12}$ was incubated with an extract of jejunum. Thus, it is apparent that the factor responsible for a change in the solubility characteristics of NHGJ-$^{57}CoB_{12}$ was contained in ileal but not jejunal extracts.

The interaction between the $^{57}CoB_{12}$ and this ileal factor appears to require NHGJ because very little free $^{57}CoB_{12}$ incubated with ileal extract coprecipitated at 15% $Na_2SO_4$ (Table II, experiment 3). In addition, if $B_{12}$-free NHGJ [3] was incubated with the ileal extract prior to the addition of NHGJ-$^{57}CoB_{12}$, only 2.4% of the radioactivity coprecipitated (Table II, experiment 4). This competitive inhibition by the $B_{12}$-free NHGJ suggests that the ileal factor which changes the solubility of NHGJ-$^{57}CoB_{12}$ involves an interaction with IF.

Extracts of isolated brush border membranes also reacted with NHGJ-$^{57}CoB_{12}$, as did the extract of the brush border–poor mucosa which was tested after separation of the brush border membranes (Table II, experiments 5 and 6). The primary purpose of this experiment was to qualitatively identify the presence of this factor on isolated brush border membranes. However, it should be noted that although there appears to be no significant difference in the percentage of radioactivity coprecipitated with either the extract of brush border membranes or the extract of brush

[3] The gastric juice contained no $B_{12}$ as determined by the $B_{12}$ radioassay method used in this laboratory.

TABLE I

*Precipitation of Free NHGJ-$^{57}CoB_{12}$ and In Vivo Labeled Ileal Extract at 15% $Na_2SO_4$**

| Experiment | Specific reactants | No. experiments | % Radioactivity precipitated at 15% $Na_2SO_4$‡ |
|---|---|---|---|
| 1 | 25 pg NHGJ-$^{57}CoB_{12}$, normal serum | 5 | 0.26 ± 0.25 |
| 2 | Labeled ileal extract§, normal serum | 4 | 56.0 ± 5.4 |
| 3 | 25 pg NHGJ-$^{57}CoB_{12}$, anti-IF antiserum | 5 | 69.5 ± 3.4 |

* The reaction mixtures contained the indicated specific reactants in the following volumes: NHGJ-$^{57}CoB_{12}$, 0.05 ml; labeled ileal extract, 0.5 ml; normal serum, 0.3 ml; anti-IF antiserum, 0.3 ml. Each mixture contained sufficient Ringer's-bicarbonate for a total volume of 2.3 ml. After 30 min, 2.3 ml of 30% $Na_2SO_4$ was added.
‡ Mean ± SE.
§ Contained 8–10 pg $^{57}CoB_{12}$.

TABLE II

*Interaction of Extracts of Ileum, Jejunum, and Brush Border Membranes with NHGJ-$^{57}CoB_{12}$**

| Experiment | Specific reactants | No. experiments | % Radioactivity precipitated at 15% $Na_2SO_4$‡ |
|---|---|---|---|
| 1 | Ileal extract, 25 pg NHGJ-$^{57}CoB_{12}$ | 7 | 48.1 ± 4.9 |
| 2 | Jejunal extract, 25 pg NHGJ-$^{57}CoB_{12}$ | 5 | 3.7 ± 1.1 |
| 3 | Ileal extract, 25 pg free $^{57}CoB_{12}$ | 4 | 4.6 ± 1.9 |
| 4 | Ileal extract,§ NHGJ (0.3 ml), 25 pg NHGJ-$^{57}CoB_{12}$ | 2 | 2.4 |
| 5 | Brush border membrane extract, 25 pg NHGJ-$^{57}CoB_{12}$ | 3 | 36.2 ± 6.9 |
| 6 | Brush border–poor extract‖, 25 pg NHGJ-$^{57}CoB_{12}$ | 3 | 27.8 ± 1.0 |

* The reaction mixtures contained the indicated specific reactants in the following volumes: extracts, 0.5 ml; NHGJ-$^{57}CoB_{12}$, 0.05 ml; $^{57}CoB_{12}$, 0.05 ml; NHGJ, 0.3 ml. Each mixture contained sufficient Ringer's-bicarbonate for a total volume of 2.0 ml. After 30 min 0.3 ml of normal serum and 2.3 ml of 30% $Na_2SO_4$ were added.
‡ Mean ± SE.
§ The ileal extract and NHGJ were preincubated for 30 min before the addition of the NHGJ-$^{57}CoB_{12}$.
‖ After the brush border membranes were separated from the homogenate by slow centrifugation the supernate was mechanically disrupted and dialyzed against Ringer's-bicarbonate solution and then tested in this experiment.

TABLE III

*Interaction of Redissolved Precipitate and Supernate of 15% $Na_2SO_4$ Fractionated Ileal Extract with NHGJ-$^{57}CoB_{12}$**

| Experiment | Specific reactants | % Radioactivity precipitated at 15% $Na_2SO_4$‡ |
|---|---|---|
| 1 | Redissolved 15% $Na_2SO_4$ precipitate, 25 pg NHGJ-$^{57}CoB_{12}$ | 41.3 |
| 2 | 15% $Na_2SO_4$ supernate, 25 pg NHGJ-$^{57}CoB_{12}$ | 8.1 |

* The ileal extract was fractionated with 150 mg/ml of anhydrous $Na_2SO_4$ and the precipitate which formed dissolved in Ringer's-bicarbonate equal to original volume. The redissolved precipitate and supernate were dialyzed against Ringer's-bicarbonate and a 0.5 ml aliquot of each incubated with NHGJ-$^{57}CoB_{12}$ for a total volume of 2.0 ml. After 30 min 0.3 ml of normal serum and 2.3 ml of 30% $Na_2SO_4$ were added.

‡ Results are mean of duplicate experiments.

border–poor mucosa, the protein content[4] of the brush border membrane extract (0.8 mg/ml) was approximately 1/12 that of the brush border–poor extract (10 mg/ml). Therefore, when considered in terms of specific activity there appears to be considerably more of this ileal factor on the brush borders than in the rest of the ileal mucosal cell.

To study the solubility properties of this ileal factor, anhydrous $Na_2SO_4$ was added directly to the ileal extract (150 mg/ml). The resulting precipitate was redissolved in Ringer's-bicarbonate and both the supernate and redissolved precipitate were dialyzed against this solution overnight at 4°C. An aliquot of each fraction was then incubated with NHGJ-$^{57}CoB_{12}$ as described previously for untreated ileal extract. Table III summarizes the results of this experiment. The ileal factor that reacts with NHGJ-$^{57}CoB_{12}$ was found primarily in the redissolved precipitate, which indicates that this factor is itself insoluble at 15% $Na_2SO_4$.

The data summarized in Table IV indicate that the interaction between NHGJ-$^{57}CoB_{12}$ and the factor in the ileal extract was inhibited at low pH and low temperature. Preheating the extract at 56°C for 30 min also significantly decreased the radioactivity which coprecipitated at 15% $Na_2SO_4$.

[4] Determined by the biuret method.

The effect of EDTA on the interaction of NHGJ-$^{57}CoB_{12}$ and the ileal factor was also investigated, and the results of these experiments are shown in Table V. Sodium EDTA but not calcium EDTA inhibited completely the interaction between NHGJ-$^{57}CoB_{12}$ and the ileal factor. The IF-$B_{12}$ complex itself was not affected by the sodium EDTA since 64.1% of the radioactivity coprecipitated at 15% $Na_2SO_4$ when the NHGJ-$^{57}CoB_{12}$ was incubated with anti-IF antiserm (experiment 3).

Experiments 4 and 5 of Table V show that when labeled extracts (prepared by in vivo incubation with NHGJ-$^{57}CoB_{12}$) were incubated with sodium EDTA only 4% of the radioactivity coprecipitated at 15% $Na_2SO_4$ yet 72.4% of the radioactivity of these very same extracts did coprecipitate at this salt concentration when Ringer's-bicarbonate was substituted for the EDTA. It is thus apparent that the in vivo interaction between NHGJ-$^{57}CoB_{12}$ and the ileal factor could be reversed in vitro by this chelating agent.

TABLE IV

*Effect of pH and Temperature on the Interaction between NHGJ-$^{57}CoB_{12}$ and the Ileal Extract*

| pH | Temperature | % Radioactivity precipitated at 15% $Na_2SO_4$* |
|---|---|---|
| | °C | |
| 2‡ | 25 | 25.0 ± 7.1§ |
| 4‡ | 25 | 50.0 ± 4.8 |
| 6‡ | 25 | 51.6 ± 4.8 |
| 8‡ | 25 | 51.1 ± 4.5 |
| 10‡ | 25 | 49.4 ± 5.4 |
| 7.2‖ | 4 | 17.6 ± 4.8¶ |
| 7.2 | 25 | 48.1 ± 7.0 |
| 7.2 | 37 | 51.7 ± 3.0 |
| 7.2 | 56, 25** | 9.7 ± 3.1 |

* Results are mean ± SE of experiments with three different extracts.

‡ The reaction mixtures contained ileal extract and NHGJ-$^{57}CoB_{12}$ in Ringer's solution (total volume 2 ml) previously adjusted to indicated pH with either 0.1 N HCl or 0.1 N NaOH. After 30 min 0.3 ml of normal serum and and equal volume of 30% $Na_2SO_4$ were added.

§ Significance compared to higher pH is $0.02 < P < 0.05$.

‖ All reactions at pH 7.2 contained the reactants in Ringer's-bicarbonate.

¶ Significance compared to 25°C and 37°C is $0.001 < P < 0.01$.

** The extract was first heated to 56°C for 30 min and the reaction mixture then incubated at 25°C.

TABLE V

*Effect of EDTA on Interaction of NHGJ-$^{57}$CoB$_{12}$ and Ileal Extract*

| Experiment | Specific reactants* | No. experiments | % Radioactivity precipitated at 15% $Na_2SO_4$‡ |
|---|---|---|---|
| 1 | Sodium EDTA, ileal extract, 25 pg NHGJ-$^{57}$COB$_{12}$ | 4 | 0 |
| 2 | Calcium EDTA, ileal extract, 25 pg NHGJ-$^{57}$CoB$_{12}$ | 4 | 49.7 ±1.8 |
| 3 | Sodium EDTA§, 25 pg NHGJ-$^{57}$CoB$_{12}$, anti-IF antiserum | 3 | 64.1 ±1.1 |
| 4 | Sodium EDTA‖, labeled ileal extract | 2 | 4.0 |
| 5 | Ringer's-bicarbonate‖, labeled ileal extract | 2 | 72.4 |
| 6 | Sodium EDTA‖, labeled ileal extract, anti-IF antiserum | 2 | 93.2 |

* Same reaction mixtures and procedure as described in footnote to Table II except that sodium or calcium EDTA was substituted for the Ringer's-bicarbonate where indicated.
‡ Mean ± SE.
§ The reaction mixture contained no ileal extract.
‖ This reaction mixture contained 0.5 ml of in vivo labeled ileal extract (8.0 pg $^{57}$CoB$_{12}$) instead of plain ileal extract and NHGJ-$^{57}$CoB$_{12}$.

Experiment 6 of Table V shows that even in sodium EDTA 93.2% of the radioactivity of these labeled extracts coprecipitated at 15% $Na_2SO_4$ when anti-IF antiserum was used in the reaction mixture, indicating that most of the radioactivity extracted from the ileum after exposing it to NHGJ-$^{57}$CoB$_{12}$ contains the IF molecule.

## Starch gel electrophoresis of labeled ileal extract

Since the results of the experiments with EDTA indicated that in vivo labeled ileal extract contained IF-bound $^{57}$CoB$_{12}$ in fractions soluble and insoluble at 15% $Na_2SO_4$, this same labeled extract was subjected to starch gel electrophoresis in order to separate and identify these fractions. Fig. 1 A illustrates the radioelectrophoretogram of labeled ileal extract subjected to electrophoresis in gel prepared in borate buffer. There were two major areas of radioactivity, one remaining at the origin (peak *A*) and one moving anodally (peak *B*). The small cathodal peak was free $^{57}$CoB$_{12}$. As shown in Fig. 1 A, 86% of the peak *A* eluate reacted with anti-IF antiserum while only 10% was insoluble at 15% $Na_2SO_4$. Similarly, 88% of

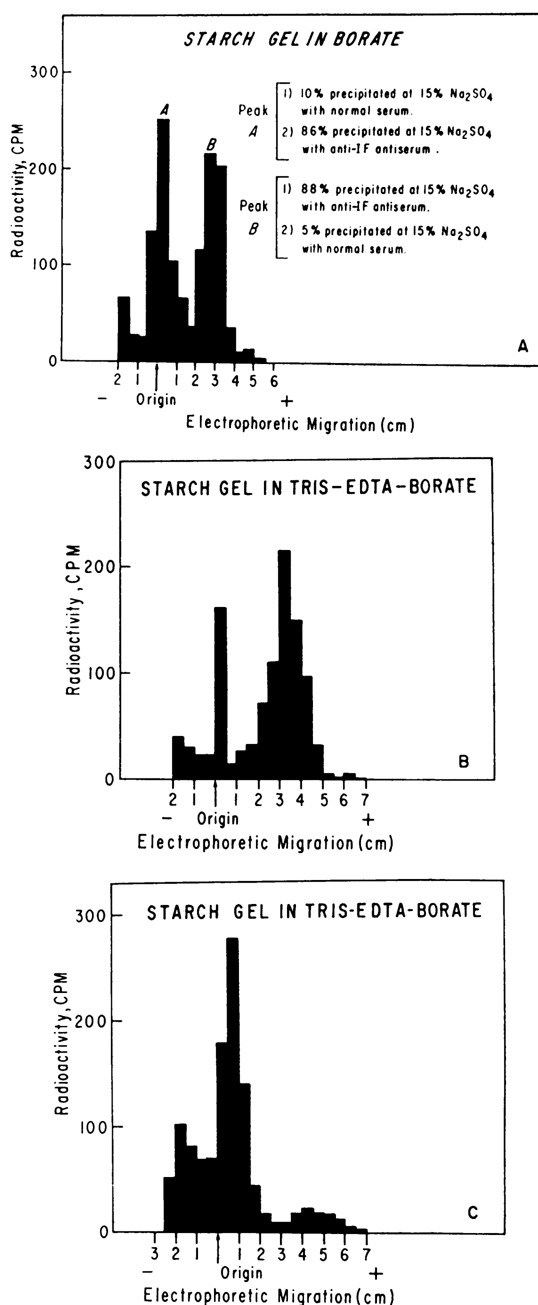


FIGURE 1 Electrophoretic patterns of labeled ileal extract subjected to starch gel electrophoresis. In Fig. 1C, the labeled extract was incubated with anti-IF antiserum prior to electrophoresis.

the peak *B* eluate reacted with anti-IF antiserum while only 5% of this radioactivity was insoluble at 15% $Na_2SO_4$. It is apparent from this study that unlike isolated human IF which moves anodally under these electrophoretic conditions

TABLE VI

*Interaction of Sephadex-Filtered, Unlabeled Ileal Extract with NHGJ-$^{57}CoB_{12}$**

| Experiment | Specific reactants | % Radioactivity precipitated at 15% $Na_2SO_4$ |
|---|---|---|
| G-100 | Excluded volume, 25 pg NHGJ-$^{57}CoB_{12}$ | 28 |
| | Inner volume, 25 pg NHGJ-$^{57}CoB_{12}$ | 0 |
| G-200 | Excluded volume, 25 pg NHGJ-$^{57}CoB_{12}$ | 40 |
| | Inner volume, 25 pg NHGJ-$^{57}CoB_{12}$ | 13 |

* The fractions constituting excluded and inner volumes were pooled, dialyzed against water, and concentrated to original volume of extract by fan evaporation at 4°C. A 0.5 ml aliquot of each fraction was then incubated with NHGJ-$^{57}CoB_{12}$ in Ringer's-bicarbonate as described in footnote to Table II.

(22), immunologically intact IF present in the ileal extract moves in two fractions with different electrophoretic mobilities. Even though the extract prior to electrophoresis contained a significant fraction of radioactivity insoluble at 15% $Na_2SO_4$, this fraction could not be positively identified after electrophoresis. The most likely explanation for this is that the $Ca^{++}$ from the extract moved to the cathode during electrophoresis and this resulted in dissociation of the IF-$^{57}CoB_{12}$ from the ileal binding factor. The smaller free IF-$^{57}CoB_{12}$ then moved anodally more rapidly, while the larger complex lagged behind probably because the dissociation occurred at a slow rate. Although an attempt was made to add calcium to the gel when it was prepared, the calcium precipitated in the cold and satisfactory electrophoretic patterns could not be obtained.

Fig. 1 B illustrates the electrophoretic pattern when the same labeled extract was run in gel prepared in Tris-EDTA-borate. A greater percentage of the total radioactivity now moved anodally compared to the pattern in Fig. 1 A, suggesting that when $Ca^{++}$ was chelated by the EDTA of the gel buffer the dissociation of IF-$^{57}CoB_{12}$ from the ileal binding factor occurred at a more rapid rate and more of the smaller, free IF-$^{57}CoB_{12}$ moved anodally.

Fig. 1 C is the electrophoretogram obtained when the same labeled extract was incubated with anti-IF antiserum prior to electrophoresis in gel prepared with Tris-EDTA-borate. Most of the radioactivity remained at the origin with gamma globulin, again indicating that the $^{57}CoB_{12}$ extracted from the ileum after incubation with NHGJ-$^{57}CoB_{12}$ is for the most part bound to IF. This method of immunologically identifying IF was described by Jeffries, Sleisenger, and Benjamin (23).

### Sephadex gel filtraton of unlabeled extract

Information regarding molecular size of this ileal binding factor was obtained by filtering an aliquot of pooled, unlabeled ileal extract through G-100 and G-200 Sephadex and reacting the concentrated filtered fractions with NHGJ-$^{57}CoB_{12}$. As shown in Table VI this factor was found in the excluded fractions of G-100 indicating that its molecular weight is probably over 100,000. Since shape as well as size influence gel filtration, particularly through G-200 Sephadex, it is tenuous to assume a molecular weight over 200,000 even though most of the factor also appeared in the excluded volume of this gel size.

### Kinetic studies of the interaction between NHGJ-$^{57}CoB_{12}$ and the ileal extract

The velocity of the reaction between the ileal binding factor and NHGJ-$^{57}CoB_{12}$ is shown in Fig. 2 A. When 25 pg of NHGJ-$^{57}CoB_{12}$ was incubated with 0.5 ml of ileal extract the increase in the amount of $^{57}CoB_{12}$ which precipitated at 15% $Na_2SO_4$ was linear for approximately 30 min. This is a rather slow reaction rate when compared to the almost instantaneous reaction between IF and $B_{12}$.

When a fixed quantity of ileal extract was incubated with an increasing quantity of NHGJ-$^{57}CoB_{12}$, the reacting sites of the ileal binding factor could be saturated, as shown in Fig. 2 B. Because this curve resembled the classical Michaelis-Menten (24) plot for enzyme-substrate interactions, the same data was analyzed by the double reciprocal Lineweaver-Burk method (25) and the result is illustrated in Fig. 2 C. From this graph the $K_m$ for NHGJ-$B_{12}$ binding by the ileal factor was calculated to be $5.5 \times 10^{-10}$ M. It would appear from this study that the reaction between this factor and IF-$B_{12}$ involves association and

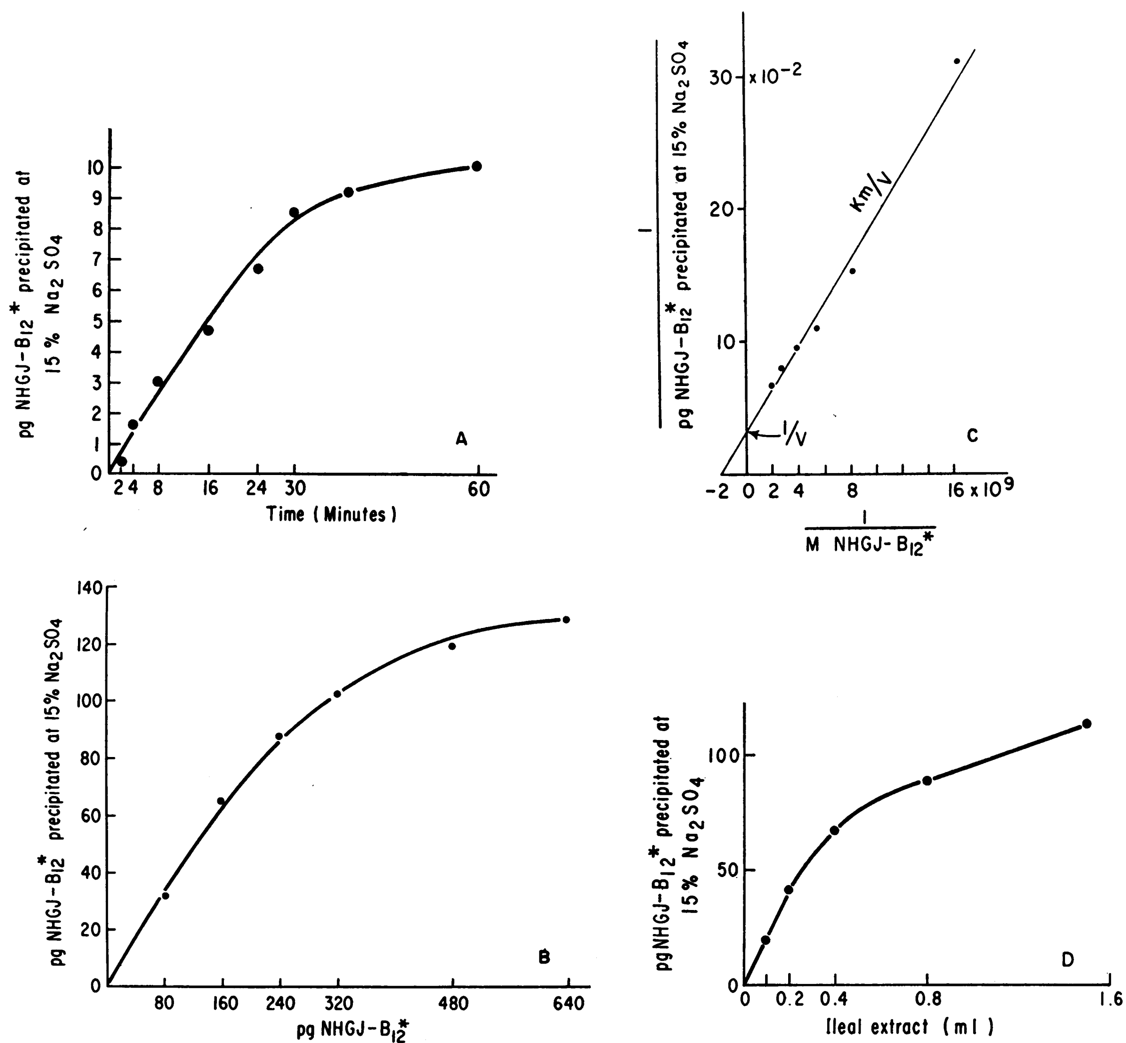


FIGURE 2 The kinetic and quantitative relationship between NHGJ-$^{57}CoB_{12}$ and ileal extract. The ordinate values indicate the pg of gastric juice-bound $^{57}CoB_{12}$ of the incubation mixtures which precipitated at 15% $Na_2SO_4$. A, The velocity of the reaction between 25 pg of NHGJ-$^{57}CoB_{12}$ and 0.5 ml of ileal extract. B, Incubation of subsaturating (less than 160 pg) and saturating (over 160 pg) concentrations of NHGJ-$^{57}CoB_{12}$ with a fixed quantity of ileal extract (0.8 ml). The NHGJ-$^{57}CoB_{12}$ values have been corrected for actual IF-$^{57}CoB_{12}$ content by determining the percentage of total radioactivity precipitated with an excess of anti-IF antiserum. C, Data in Fig. 2B plotted by the Lineweaver-Burk method. D, The quantitative relationship between an increasing concentration of ileal extract and a fixed concentration of NHGJ-$^{57}CoB_{12}$ (140 pg).

dissociation constants as do enzyme-substrate complexes, and that as the intermediary binding mechanism(s) becomes saturated the reaction reaches a maximum velocity.

An unexpected finding was observed when a fixed concentration of NHGJ-$^{57}CoB_{12}$ was incubated with an increasing concentration of ileal extract. Instead of finding all the IF-$^{57}CoB_{12}$ bound to the ileal factor when there was an excess of ileal extract, the amount of IF-$^{57}CoB_{12}$ which precipitated at 15% $Na_2SO_4$ also approached an asymptote as shown in Fig. 2D. This finding suggests that an additional cofactor in NHGJ may be necessary for the binding of IF to this ileal factor, and that as this cofactor is depleted or saturated, proportionately less of the IF-$B_{12}$ will be bound even when there is an excess of ileal extract.

## DISCUSSION

The results of the studies presented in this report indicate that a macromolecular factor which binds human intrinsic factor can be extracted from the distal ileum and not the jejunum of the guinea pig. Although the exact nature of this substance has not been defined, it is most probably a protein of rather high molecular weight since it is nondialyzable, is excluded from G-200 Sephadex gel, is precipitated at 15% $Na_2SO_4$, and is heat- and acid-labile.

The evidence that this ileal factor is a binder of IF and not $B_{12}$ alone are the results of the experiments which demonstrated that immunologically intact IF was identified in fractions of ileal extract with different solubility and electrophoretic properties after the intact ileum was incubated with NHGJ-bound vitamin $B_{12}$. Additional support for this is the observation that the in vitro interaction between NHGJ-$^{57}CoB_{12}$ and the ileal extract could be competitively inhibited by an excess of $B_{12}$-free NHGJ.

Some of the properties observed for this IF-binding factor would account for the conditions which influence the absorption of $B_{12}$ described previously by other investigators. It is well established that chelation of calcium by EDTA inhibits IF activity in vitro (8, 9, 12) and in vivo (26, 27). The inhibition of the formation of a complex between the ileal extract and NHGJ-$^{57}CoB_{12}$ by sodium EDTA and its reversal by calcium EDTA are consistent with these observations (Table V). The inhibitory effect of low temperature (5, 9) and low pH (12) on the in vitro activity of IF are similar to the results of the experiments described in this report which demonstrate that these conditions inhibited the binding of IF-$^{57}CoB_{12}$ by the ileal extract (Table IV).

The identification of the IF-binding factor in extracts of isolated brush border membranes suggests that it may be localized to this site of the epithelial cell, particularly since much more binding factor was found per mg of protein in the border membrane extract than in the extract of the brush border–poor mucosa. Such a localization of this binding factor is consistent with the observations of Donaldson, Mackenzie, and Trier (28) that human gastric juice enhanced the uptake of $B_{12}$ to epithelial cell brush borders and microvillous membranes of the distal small intestine of the hamster. Mackenzie, Kopp, Donaldson, and Trier (29) recently reported that antiserum produced against isolated brush border preparations inhibited the uptake of vitamin $B_{12}$. If the primary location of this ileal binding factor is on this site of the mucosal cell, the antibody-induced impaired $B_{12}$ absorption may very well have been due to specific antibody inactivation of this factor.

Hansen and Miller (30) reported in an abstract that RNA competitively inhibited the binding of IF-$B_{12}$ to zirconyl phosphate gel and suggested that this nucleic acid may be a cofactor requirement for vitamin $B_{12}$ absorption. Although it is possible that the ileal IF-binding factor described in this report may be RNA or an RNA nucleoprotein, its stability at pH 10 (Table IV) where both RNA and RNA protein are generally unstable makes this contention unlikely. However, more definite conclusions concerning the exact chemical nature of this ileal protein must await its purification.

Cooper (14) has studied the uptake of $B_{12}$ by everted sacs of guinea pig intestine and also found Michaelis-Menten kinetics when saturating quantities of NHGJ-$B_{12}$ were used. It is interesting that he obtained a $K_m$ value for $B_{12}$ uptake by the intestinal sac of $6.8 \times 10^{-10}$ M which is within close agreement to the $5.5 \times 10^{-10}$ M value obtained in this study. However, it is now more apparent that these constants really reflect not $B_{12}$ uptake by intestine or intestinal extracts but rather the binding of the IF-$B_{12}$ complex by this ileal binding factor (or perhaps more appropriately termed ileal intrinsic factor). The importance of the $B_{12}$ molecule in this reaction must still be studied. Although a 120-fold excess of NHGJ did competitively inhibit the binding of NHGJ-$B_{12}$ by the ileal extract (Table II), Cooper (14) has reported that a four fold excess of free NHGJ did not compete as effectively as NHGJ-$B_{12}$ for the receptor sites of guinea pig ileum. A more precise investigation into the kinetics of the reaction between this ileal factor, NHGJ-$B_{12}$, and $B_{12}$-free NHGJ will also require a more purified preparation of this macromolecule.

Although direct extrapolation of the results of animal experiments to explain human biologic phenomena may well be questioned, the results of the experiments described in this report indicate that

an IF-binding factor is probably also present in human ileum for two principle reasons. First, it has been shown that the IF-enhanced absorption of $B_{12}$ in guinea pig ileum can be mediated by many sources of animal IF (31) which suggests that this is a fundamental mechanism common to all mammalian species. Secondly, and more specifically, it has been shown that human IF enhances the absorption of $B_{12}$ by guinea pig ileum (9). Since, for obvious reasons, it was not possible to use normal human ileum, the combination of NHGJ and guinea pig ileum, therefore, appeared to be most appropriate.

Recently, Frentz, Miller, and Hansen (32) reported in an abstract that 4 hr after the administration of IF-$B_{12}$ to the rat, the soluble radioactivity of the intestinal homogenate was associated with two binding proteins, one of molecular size comparable to IF-$B_{12}$ (50,000–60,000) and a second with a mol wt greater than 100,000. Although they did not report further identification of these $B_{12}$-protein complexes, it seems probable from the guinea pig experiments described in this report that one complex was IF-$B_{12}$ and the second may have been IF-$B_{12}$ bound to the ileal binding factor. These observations in the rat as well as the results of our experiments with guinea pig ileal extracts, support the premise that IF binding by an ileal protein is a fundamental mechanism of $B_{12}$ absorption, probably common to all mammalians.

As the mechanism for vitamin $B_{12}$ absorption unfolds, it clearly involves the formation of at least two intermolecular complexes, each serving a specific function. The formation of the IF-$B_{12}$ complex is probably an evolutionary development to protect the vitamin from intestinal microbial degradation, since several bacteria including forms of *Aerobacter* (33) and *Pseudomonas* (34) are known to convert $B_{12}$ to other pigments. The protective effect of IF binding is also known since the original observations of Ternberg and Eakin (2) demonstrated that binding of $B_{12}$ to gastric juice rendered it unavailable as a growth promoting factor for microorganisms.

Once the vitamin has been transported to the site of absorption in the distal ileum, the large size of the $B_{12}$ molecule prevents passive diffusion and necessitates a time consuming absorptive process which the rate of intestinal flow in this area would preclude. Accordingly, a second intermolecular complex forms in which IF with its attached $B_{12}$ moiety binds to an ileal intrinsic factor thus preventing its loss into the lumen of the colon. Although this speculative role of the ileal binding factor as an anchor for the IF-$B_{12}$ complex may not be consistent with the relatively slow rate of in vitro interaction of these two factors found in these studies, the higher concentration of IF-$B_{12}$ in intestinal succus and an in vivo milieu may result in a significantly more rapid rate of binding of the IF-$B_{12}$ complex.

Another question which must ultimately be answered is whether, in addition to this speculative role as an anchor for IF-$B_{12}$, the ileal binding factor facilitates the entry of $B_{12}$ into and through the mucosal cell. We are now studying methods of separating more precisely the cellular constituents of the intestinal mucosa using density gradient centrifugation. If it can be shown that this IF-binding factor is present in the extract of mucosa completely free of brush border contamination, it would indicate that it serves more than just a passive IF-binding role, and participates actively as a carrier of the IF-$B_{12}$ complex from cell surface to cell cytoplasm.

## ACKNOWLEDGMENTS

I would like to thank Doctor Robert Goldstein for his helpful suggestions during the course of this investigation and for his advice in preparing the manuscript.

I would like to acknowledge the technical assistance of Valerie Ger, a medical student at New York Medical College, who worked in the laboratory during a tenure period of summer research.

This work was supported by grant No. AM 06045 from the United States Public Health Service. Dr. Rothenberg is a Career Investigator of the New York City Health Research Council, contract No. I-423.

## REFERENCES

1. Castle, W. B. 1929. Observations of the etiologic relationship of achylia gastrica to pernicious anemia: I. The effect of administration to patients with pernicious anemia of the contents of the normal human stomach recovered after the ingestion of beef muscle. *Am. J. Med. Sci.* **178:** 748.
2. Ternberg, J. L., and R. E. Eakin. 1949. Erythein and apoerythein and their relation to anti-pernicious anemia principle. *J. Am. Chem. Soc.* **71:** 3858.
3. Booth, C. C., and D. L. Mollin. 1957. Importance of the ileum in the absorption of vitamin B12. *Lancet.* **2:** 1007.
4. Booth, C. C., I. Chanarin, B. B. Anderson, and D. L. Mollin. 1957. The site of absorption and tissue distri-

bution of orally administered $^{58}$Co-labeled vitamin B12 in the rat. *Brit. J. Haemat.* **3**: 253.
5. Strauss, E. W., T. H. Wilson, and A. Hotchkiss. 1960. Factors controlling B12 uptake by intestinal sacs in vitro. *Am. J. Physiol.* **198**: 103.
6. Booth, C. C., and D. L. Mollin. 1956. Plasma, tissue, and urinary radioactivity after oral administration of $^{56}$Co-labeled vitamin B12. *Brit. J. Haemat.* **2**: 223.
7. Okuda, K. 1962. Mucosal adsorption and absorption of vitamin B12 in the intestine of the rat. *Proc. Soc. Exptl. Biol. Med.* **111**: 320.
8. Cooper, B. A., and W. B. Castle. 1960. Sequential mechanisms in the enhanced absorption of vitamin B12 by intrinsic factor in the rat. *J. Clin. Invest.* **39**: 199.
9. Cooper, B. A., W. Paranchych, and L. Lowenstein. 1962. Studies on the absorption by guinea pig intestine of cyanocobalamin incubated with intrinsic factor. *J. Clin. Invest.* **41**: 370.
10. Nieweg, H. O., S. C. Shen, and W. B. Castle. 1957. Mechanism of intrinsic factor action in the gastrectomized rat. *Proc. Soc. Exptl. Biol. Med.* **94**: 223.
11. Abels, J., J. J. M. Vegter, M. G. Woldring, J. H. Jans, and H. O Nieweg. 1959. The physiologic mechanism of vitamin B12 absorption. *Acta Med. Scand.* **165**: 105.
12. Herbert, V. 1959. Mechanism of intrinsic factor action in everted sacs of rat small intestine. *J. Clin. Invest.* **38**: 102.
13. Boass, A., and T. H. Wilson. 1964. Intestinal absorption of intrinsic factor and B12-intrinsic factor complex. *Am. J. Physiol.* **207**: 27.
14. Cooper, B. A. 1964. The uptake of $Co^{57}$-labeled vitamin B12 by everted sacs of intestine in vitro. *Medicine.* **43**: 689.
15. Ukyo, S., and B. A. Cooper. 1965. Intrinsic factor-like activity in extracts of guinea pig intestine. *Am. J. Physiol.* **208**: 9.
16. Kay, A. W. 1953. Effects of large doses of histamine on gastric secretion of HCl. An augmented histamine test. *Brit. Med. J.* **2**: 77.
17. Rothenberg, S. P. 1961. Assay of serum vitamin B12 concentration using $Co^{57}$-B12 and intrinsic factor. *Proc. Soc. Exptl. Biol. Med.* **108**: 45.
18. Rothenberg, S. P. 1966. A radioimmunoassay for human intrinsic factor. *J. Lab. Clin. Med.* **67**: 879.
19. Miller, D., and R. K. Crane. 1961. A procedure for the isolation of the epithelial brush border membrane of hamster small intestine. *Anal. Biochem.* **2**: 284.
20. Lau, K. S., C. Gottlieb, L. R. Wasserman, and V. Herbert. 1965. Measurement of serum vitamin B12 level using radioisotope dilution and coated charcoal. *Blood.* **26**: 202.
21. Smithies, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61**: 629.
22. Rothenberg, S. P. 1966. Immunologic isolation of human intrinsic factor. *Proc. Soc. Exptl. Biol. Med.* **122**: 1.
23. Jeffries, G. H., M. H. Sleisenger, and L. L. Benjamin. 1963. The immunologic identification and quantitation of human intrinsic factor in gastric secretions. *J. Clin. Invest.* **42**: 442.
24. Michaelis, L., and M. L. Menten. 1913. Die Kinetik der Invertinwirkung. *Biochem. Z.* **49**: 333.
25. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* **56**: 658.
26. Gräsbeck, R., and W. Nyberg. 1958. Inhibition of radiovitamin B12 absorption by ethylenediaminetetraacetate (EDTA) and its reversal by calcium ions. *Scand. J. Clin. Lab. Invest.* **10**: 448.
27. Okuda, K., and K. Sasayama. 1965. Effects of ethylenediaminetetraacetate and metal ions in intestinal absorption of vitamin B12 in man and rats. *Proc. Soc. Exptl. Biol. Med.* **120**: 17.
28. Donaldson, R. M., Jr., I. L. Mackenzie, and J. S. Trier. 1967. Intrinsic factor-mediated attachment of vitamin B12 to brush borders and microvillous membranes of hamster intestine. *J. Clin. Invest.* **46**: 1215.
29. Mackenzie, I. L., W. L. Kopp, R. M. Donaldson, and J. S. Trier. 1967. Antibodies to intestinal microvillous membranes: Inhibition of intrinsic factor mediated attachment of vitamin B12 to hamster brush borders. *Clin. Res.* **15**: 239. (Abstr.)
30. Hansen, H. S., and O. N. Miller 1960. On the mechanism of interaction of intrinsic factor (IF) and cofactor. *Federation Proc.* **19**: 418. (Abstr.)
31. Wilson, T. H., and E. W. Strauss. 1959. Some species differences in the intrinsic factor stimulation of B12 uptake by small intestine in vitro. *Am. J. Physiol.* **197**: 926.
32. Frentz, G. D., O. N. Miller, and H. J. Hansen. 1966. Characterization of vitamin B12 binders in rat intestine after oral administration of rat intrinsic factor-$B12Co^{57}$ complex. *Clin. Res.* **14**: 432. (Abstr.)
33. Helgeland, K., J. Jonsen, S. Laland, T. Lygreen, and O. Rømcke. 1963. Biological activity of a brownish-yellow pigment produced from vitamin B12 by *Aerobacter aerogenes. Nature.* **199**: 604.
34. Burgus, R. C., J. B. Hufham, W. M. Scott, and J. J. Pfiffner. 1964. Microbial degradation of corrinoids. III. Pigments derived from vitamin B12 by *Pseudomonas rubescens. J. Bacteriol.* **88**: 1139.