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The 1966 recipient of the Meltzer Award was Dr. Max D. Cooper, Department of Pediatrics, University of Minnesota College of Medicine. The selection was made by a committee appointed by the President consisting of Doctors S. E. Bradley, J. L. Melnick, and I. H. Page.

SECTION MEETINGS

DISTRICT OF COLUMBIA	
Naval Medical Research Institute	March 17, 1966
CLEVELAND	
Lakeside Hospital	February 21, 1966
Western Reserve University	April 25, 1966
IOWA	
University of Iowa	April 26, 1966
MINNESOTA	
St. Paul Campus, University of Minnesota	March 17, 1966

Immunologic Isolation of Human Intrinsic Factor.* (31035)

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Taylor(1) and Schwartz(2) have shown that serum from some patients with pernicious anemia (PA) will inhibit the intrinsic factor (IF)-mediated absorption of vitamin B_{12}. Such sera can also *block* the *in vitro* binding of B_{12} by IF(3,4,5) and also *bind* the IF-B_{12} complex(6). There is sufficient evidence to indicate that this *blocking* and *binding* property of PA serum is antibody-mediated for it can be localized to the gamma globulin fraction of the serum(7,6). This report will describe the methodology employed to isolate IF from human gastric juice using the IF-*binding* property of a serum obtained from a patient with PA.

Methods. Human gastric juice was collected 30 minutes after augmented histamine stimulation(8) from a normal subject and adjusted first to pH 9.0 with 0.1 N NaOH for 15 minutes to inactivate pepsin, and then to pH 7.0 with 0.1 N HCl. Solid debris and mucus were removed by high speed refrigerated centrifugation and the clear supernatant solution was stored at −20° until processed.

The initial step in the isolation of IF was the labeling of the gastric juice with $Co^{57}B_{12}$ (B_{12}*), with a specific activity of 15 $\mu c/\mu g$.‡ As will be discussed subsequently, this step was important in protecting the IF from damage during the isolation procedure. Two levels of B_{12}* saturation were employed. To follow the *in vitro* procedures, 5 $m\mu g$ of B_{12}* was used to label 1 ml of gastric juice. When sufficient IF-B_{12}* was needed for *in vivo* absorption studies, 30 $m\mu g$ of B_{12}* was used to label 1 ml of gastric juice.

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Radioactivity was assayed in a 2-inch well-type thallium activated sodium iodide crystal in combination with a gamma ray spectrometer and scaler. For the absorption studies performed by the urinary excretion method of Schilling(9) the radioactivity of a 15 ml sample of urine was assayed in a 3-inch well-type scintillation crystal. Sufficient counts were recorded for a counting error of less than 3%.

The procedure used to isolate the B_{12}*-labeled IF from the gastric juice may be divided into two steps: (1) binding of the IF by the antibody and separation of the antibody-IF (Ab-IF) complex from the gastric juice; (2) separation of the IF from the Ab-IF complex.

Preparation of the Ab-IF complex. A 1 ml aliquot of the previously prepared gastric juice was incubated with B_{12}* (either 5.0 or 30 mμg) for 30 minutes at room temperature. This was followed by addition of 7.5 ml of the antiserum and the incubation continued for an additional 30 minutes with gentle agitation. The Ab-IF complex was separated from this mixture by precipitating the gamma globulin with tepid 30% sodium sulfate. In the absence of serum, a mixture of equal volumes of 30% sodium sulfate and gastric juice did not result in any visible precipitate. The precipitate containing the Ab-IF complex was washed twice with 15% sodium sulfate, redissolved in 7.5 ml of 0.9% sodium chloride, and dialyzed overnight against 4 liters of this salt solution at 4°. Following dialysis the Ab-IF complex was frozen until treated for the separation of the IF.

When normal serum was substituted for the antibody containing PA serum, no radioactivity was precipitated by the addition of 30% sodium sulfate. Therefore, it is apparent that IF not bound to antibody, and non-IF B_{12} binding proteins were not co-precipitated by the precipitation of non-specific gamma globulin. In addition the gastric juice of 2 patients with congenital absence of IF but with otherwise normal gastric secretory function failed to react with this PA serum used to isolate the IF(10); it is, therefore, presumed that this antiserum contains an antibody(s) directed only against IF and no other B_{12}-binding protein.

Separation of the IF from Ab-IF complex. When sufficient IF was needed for *in vivo* absorption studies the whole post dialysis volume of Ab-IF was processed as described below. For other *in vitro* studies usually 1 or 2 ml of the sample was so treated. The basic procedure followed was a short period of acidification at 0° to separate the IF from the antibody, and then selective precipitation of the gamma globulin leaving the free IF in the supernatant solution. Accordingly, the Ab-IF solution was acidified at 0° by addition of an equal volume cold 0.1 N HCl. After 4.5 minutes, cold 50% ethyl alcohol was added and the pH raised to 6.0 to 7.0 with cold (0°) 0.1 N NaOH in order to precipitate the gamma globulin. The volume of base required usually equalled the volume of acid previously used. The volume of ethyl alcohol employed was equal to the total volume of sample, acid, and anticipated volume of base, so that the final precipitating concentration was 25%. The supernatant solution containing the free IF was separated from the precipitated gamma globulin by refrigerated centrifugation. The precipitate was redissolved in a volume of 0.9% sodium chloride equal to the initial volume of Ab-IF and the process repeated. Usually the precipitate did not dissolve in the sodium chloride to form a clear solution until acidified with the 0.1 N HCl. Repeating this procedure 4 times separated approximately 80-85% of the IF from the antibody and 85-90% of the IF so obtained could be rebound by new antiserum. Since work in this laboratory has shown that boiled IF failed to react with antibody(10), the criterion followed for estimating the structural integrity of the IF recovered was its immunologic reactivity, that is, the percent of radioactivity separated from the antibody which could be rebound by new antiserum. The results of a typical separation are summarized in Table I.

All the supernatant fractions following such a quadruplicate acid-alcohol treatment were pooled and dialyzed in the cold against 2 changes of 4 liters each of distilled water for 24 hours. When the gastric juice was saturated with 30 mμg of B_{12}* prior to separa-

tion, approximately 20 mμg were recovered with the isolated IF. One portion of this material was lyophilized and reconstituted with 0.9% sodium chloride and subjected to starch gel electrophoresis(11) at 8.75 volts/cm for 16 hours using a tris-EDTA-borate buffer (0.1 M:4.2 × 10^{-4} M:0.015 M), pH 9.1. The gel was cut in 0.5 cm segments, placed into test tubes and the radioactivity assayed in the well scintillation detector. Another aliquot of the saline-reconstituted, lyophilized sample was applied to Sephadex G-100 and G-200 columns and eluted with 0.9% sodium chloride.

Results. Fig. 1 is the radioelectrophoretogram obtained when the isolated IF fraction was subjected to starch gel electrophoresis. A

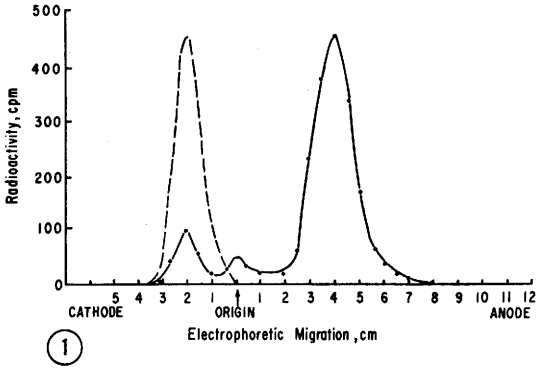
TABLE I. Effect of Sequential Acidification and Alcohol Precipitation on Antibody-Intrinsic Factor (Ab-IF) Complex.*

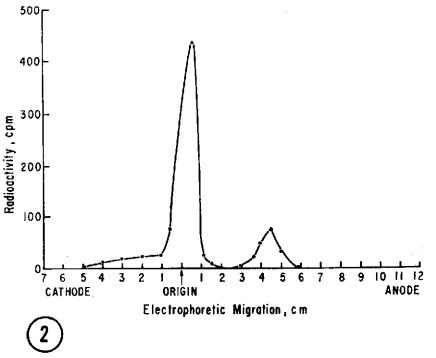
Initial radioactivity of Ab-IF = 4434 cpm

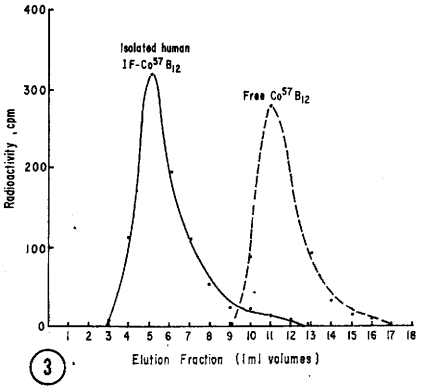
Sequential acid-alcohol treatment	Radioactivity in supernate (free IF)	% IF separated from antibody	% Free IF rebound by new antibody†
1	2531	57.0	85
2	594	13.3	84
3	342	7.7	90
4	163	3.6	91
Total	3630	81.6	

* The IF has been labeled with $Co^{57}B_{12}$.

† Each fraction was dialyzed against distilled water, concentrated by lyophilization, and then incubated with new antiserum. The radioactivity precipitated with the gamma globulin upon addition of sodium sulfate represents the amount of IF rebound by antibody.







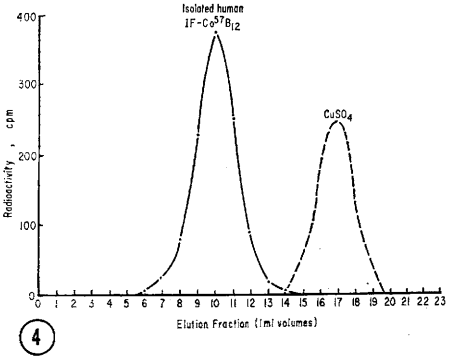


FIG. 1. Radioelectrophoretogram of isolated IF-$Co^{57}B_{12}$ (solid line) and free $Co^{57}B_{12}$ (interrupted lines) after electrophoresis on starch gel.

FIG. 2. Radioelectrophoretogram of the isolated IF-$Co^{57}B_{12}$ subjected to starch gel electrophoresis after incubation with anti IF antiserum. Major peak of activity remains at the origin corresponding to the area of gamma globulin migration. The small anodal peak of activity is probably free $Co^{57}B_{12}$ which is now bound to a serum protein.

FIG. 3. Radiochromatogram of the isolated IF-$Co^{57}B_{12}$ complex eluted from a 16 cm x 1 cm Sephadex G-100 column with 0.9% sodium chloride.

FIG. 4. Radiochromatogram of the isolated IF-$Co^{57}B_{12}$ complex eluted from a 27 x 1 cm Sephadex G-200 column with 0.9% sodium chloride.

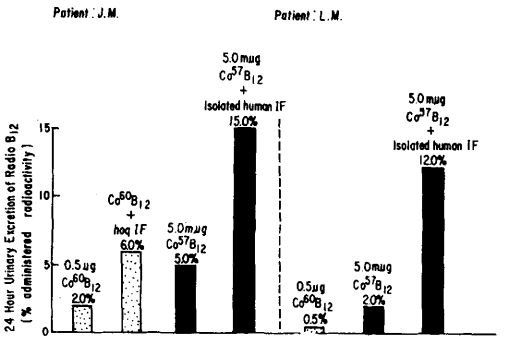


FIG. 5. Urinary excretion of 5 mμg of $Co^{57}B_{12}$ alone and with isolated IF in 2 patients with documented PA. Previous studies with 0.5 μg of $Co^{60}B_{12}$ are also shown.

small amount of radioactivity moved cathodally, corresponding to the movement of free B_{12}*, and was probably the result of some dissociation from IF during the lyophilization process. A small amount of radioactivity remained at the origin and probably represented some denatured IF-B_{12} complex. The major peak of radioactivity moved anodally and represented the IF-B_{12} complex. As shown in Fig. 2, if the sample of IF was incubated with new antiserum prior to electrophoresis the major peak of radioactivity remained at the origin overlying the gamma globulin. These findings are similar to those reported by Jeffries and coworkers(6) when they subjected IF incubated with inhibitory PA serum to starch gel electrophoresis. Fig. 3 and 4 are the radiochromatograms obtained when a sample of isolated IF was applied to Sephadex G-100 and G-200 columns, respectively. The major portion of radioactivity appeared in the outer volume of each, and distinctly separate from free B_{12}* and copper sulfate, which came off the column in a later elution volume. If artifactual or spontaneous polymerization of the IF molecule has not occurred, these findings would suggest that the IF-B_{12} complex may have a molecular weight greater than 200,000.

The most significant finding supporting the functional integrity of the isolated IF was its ability to enhance the absorption of 5 mμg of $Co^{57}B_{12}$ in 2 patients with PA. The urinary excretion of $Co^{57}B_{12}$ following oral administration of approximately 5 mμg alone, and bound to the isolated IF is shown in Fig. 5. For comparative purposes the absorption of 0.5 μg of $Co^{60}B_{12}$ was also measured, and in patient J.M., the effect of hog IF was studied. In patient J.M., the excretion of $Co^{57}B_{12}$ was 5.0% when administered alone and increased to 15% when administered bound to the isolated IF. In patient L.M. the excretion of the $Co^{57}B_{12}$ was 2.0% when administered alone and rose to 12.0% when administered with the isolated IF.

Discussion. It is evident from these findings that it is possible to isolate immunologically a B_{12}-binding protein from human gastric juice which has intrinsic factor activity. This protein moves anodally on starch gel electrophoresis, elutes in the outer volume from Sephadex G-100 and G-200 columns and enhances the intestinal absorption of radio B_{12}.

When this procedure to isolate IF is employed with B_{12}-free gastric juice, material with B_{12} binding property has not been isolated. The damaging effect of this procedure on B_{12}-free IF is due to the alcohol and not the acid. Table II summarizes the results of an experiment where B_{12}-free gastric juice was acidified with HCl for 30 minutes. There was no significant effect on either the B_{12} binding capacity of the gastric juice or the immunologic reactivity of IF. Table III summarizes the results of a similar experiment

TABLE II. Effect of Acidification on B_{12} Binding Capacity and Antibody Reacting Sites of Gastric Juice.

Incubation of gastric juice with*	B_{12} binding capacity (mμg/ml)	% Radioactivity bound by antiserum†
1. Veronal buffer	32	42
2. .5 N HCl (pH of mixture 1.4)	31	39

* 1 ml of gastric juice was incubated with 0.2 ml veronal buffer and with 0.2 ml of 0.5 N HCl for 30 min. Each was then diluted 1:4 in veronal buffer and 50 μl of each was incubated with 1000 μμg of $Co^{57}B_{12}$ in veronal buffer. The percent radioactivity bound determined by precipitation with $ZnSO_4$ and $Ba(OH)_2$ (ref. 12) multiplied by the dilution factor (80) is the B_{12} binding capacity.

† 50 μl of each 1:4 dilution was incubated with 400 μμg of $Co^{57}B_{12}$ in veronal buffer for 15 min, following which antiserum was added and the mixture incubated for 30 min. The gamma globulin was precipitated with an equal volume of 30% Na_2SO_4 and the radioactivity of the supernate assayed.

TABLE III. Effect of Ethyl Alcohol on B_{12} Binding Capacity and Antibody Reacting Sites of Gastric Juice.

Incubation of gastric juice with*	B_{12} binding capacity (mμg/ml)	% Radioactivity bound by antiserum†
1. .9% NaCl	24	60
2. 25% ethyl alcohol	12.5	41

* 1 ml of gastric was incubated with 1 ml 0.9% NaCl and with 1 ml of 50% ethyl alcohol for 30 min and each mixture then dialyzed against cold water; 1 ml of a 1:40 dilution of each was then incubated with 1000 $\mu\mu$g $Co^{57}B_{12}$. The percent radioactivity bound, determined by precipitation with $ZnSO_4$ and $Ba(OH)_2$ (ref. 12), multiplied by the dilution factor (40) is the B_{12} binding capacity.

† 1 ml of each 1:40 diluted sample was incubated with 500 $\mu\mu$g $Co^{57}B_{12}$ and then with 1 ml of antiserum. The gamma globulin was precipitated with an equal volume of 30% Na_2SO_4 and the radioactivity of the supernate assayed.

where another sample of gastric juice was incubated with 25% ethyl alcohol. In this instance the B_{12} binding capacity of the gastric juice decreased from 24 to 12.5 mμg/ml, and the percent of B_{12} binding protein in the gastric juice bound by antiserum dropped from 60% to 41%.

Theoretically, if the antiserum contained only an antibody to IF and to no other gastric juice protein, the IF isolated should be pure. However, the antiserum may have contained antibodies to gastric juice proteins which are not B_{12} binding. In addition, the precipitation of gamma globulin with sodium sulfate may have co-precipitated other gastric juice proteins even though the sodium sulfate in the absence of serum did not produce visible precipitation.

When sufficient material is isolated to permit protein determination, it will be possible to estimate the purity of the isolated IF by comparing the actual quantity of protein recovered to the quantity expected if each mole of IF of an assumed molecular weight (50,000, 100,000 or 200,000) bound one mole of B_{12}. A close agreement between the figures would indicate that a pure IF fraction has been isolated. Conversely, contamination with other proteins would increase the actual recovery over that expected. With a greater amount of material isolated it will also be possible to establish purity by additional studies employing zone and immuno-electrophoresis, chromatography and ultracentrifugation.

With the antiserum employed for this study, it would take several thousand milliliters to isolate 1 mg of IF. Since this is most impractical, the possibility of reutilizing the gamma globulin to combine with new IF is under investigation. Although the acid-alcohol treatment of the antibody-IF complex changes the solubility characteristics of the gamma globulin, for it goes back into solution only at a more alkaline pH, preliminary studies have indicated that many antibody reacting sites have remained intact since they will again combine with IF.

Summary. This report describes a procedure whereby IF is separated from human gastric juice by reacting it with specific human anti-human IF antibody contained in the serum of a patient with PA. Following separation of the antibody-IF complex from the rest of the gastric juice and serum, the IF was separated from the antibody by repetitive acidifications and selective precipitation of the gamma globulin. The separated IF still retained its immunologic reactivity, moved electrophoretically toward the anode, eluted from Sephadex G-100 and G-200 columns in the outer volume, and most important, enhanced the intestinal absorption of vitamin B_{12} in 2 patients with pernicious anemia.

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