

# RADIOASSAY OF SERUM VITAMIN B<sub>12</sub> BY QUANTITATING THE COMPETITION BETWEEN Co<sup>57</sup>B<sub>12</sub> AND UNLABELED B<sub>12</sub> FOR THE BINDING SITES OF INTRINSIC FACTOR

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RADIOASSAY OF SERUM VITAMIN B<sub>12</sub> BY QUANTITATING THE  
COMPETITION BETWEEN Co<sup>57</sup>B<sub>12</sub> AND UNLABELED B<sub>12</sub>  
FOR THE BINDING SITES OF INTRINSIC FACTOR \*

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The extremely low concentration of vitamin B<sub>12</sub> in normal and B<sub>12</sub>-deficient blood has, heretofore, necessitated the use of microbiologic methods for its assay. Such bioassays are not only indirect and time consuming, but also require particular bacteriologic skills. In an earlier report from this laboratory (1) preliminary data were presented describing a simplified method for assaying this vitamin based on the competition between unlabeled vitamin B<sub>12</sub> and isotopically labeled vitamin B<sub>12</sub> for the binding sites of intrinsic factor.

This investigation was prompted by the observation that the electrophoretic mobility of Co<sup>60</sup>B<sub>12</sub> could be altered by prior incubation with intrinsic factor (IF). Similar findings had been made by Schilling and Deiss (2) using human gastric juice instead of IF. Figure 1 is a diagrammatic illustration of radioelectrophoretograms of free and IF-bound Co<sup>60</sup>B<sub>12</sub>. Whereas free Co<sup>60</sup>B<sub>12</sub> has essentially no electrophoretic mobility (Figure 1A), when bound to IF, it migrates toward the anode (Figure 1B). If the IF is incubated with a mixture of Co<sup>60</sup>B<sub>12</sub> and unlabeled B<sub>12</sub>, less of the Co<sup>60</sup>B<sub>12</sub> will be bound because of the competition now present for the binding sites of IF. Two peaks of radioactivity appear, the free unbound Co<sup>60</sup>B<sub>12</sub> at the origin and that bound to the IF (Figure 1C).

These findings are similar to those reported by Berson and co-workers (3) on the binding of insulin-I<sup>131</sup> by specific anti-insulin antibodies in studies employing paper electrophoresis. As they exploited their observations in developing an immunoassay for insulin (4, 5), so it seemed possible that the inverse relationship between the ratio of bound to free Co<sup>60</sup>B<sub>12</sub> and total concen-

tration of unlabeled B<sub>12</sub> might be similarly applied to the assay of cyanocobalamin.

These initial observations were made using millimicrogram to microgram quantities of Co<sup>60</sup>B<sub>12</sub> with proportional amounts of IF sufficient for binding. Similar use of paper electrophoresis to separate free and bound B<sub>12</sub> for serum assay proved inexpedient because the physiologic concentration of this vitamin is so low (in the microgram per milliliter range) that the corresponding levels of radioactivity, with the available specific activity of isotopically labeled B<sub>12</sub>, would be difficult if not impossible to detect by paper-strip scanning. To overcome this difficulty, a method employing precipitation to separate the IF-B<sub>12</sub> complex from free B<sub>12</sub> was sought. In this way, quantities of solution greater than could be applied to paper strips could be counted in a well-type scintillation detector. Barium hydroxide-zinc sulfate protein precipitation was found to precipitate only protein-bound B<sub>12</sub>, leaving unbound B<sub>12</sub> in solution.

The assay as originally described (1), employing intrinsic factor as the binding protein, presented one major difficulty. The B<sub>12</sub>-binding property of IF appeared to be greater in the serum extract to be assayed for B<sub>12</sub> as compared to the aqueous solution of crystalline B<sub>12</sub> used as the standard. It was, therefore, necessary to use the extract of B<sub>12</sub>-deficient serum as the diluent for the standard. This problem has been corrected, and the original method significantly modified. This report describes in detail the assay as now carried out.

#### MATERIALS AND METHODS

Co<sup>57</sup>B<sub>12</sub> is used as the isotopically labeled B<sub>12</sub> because it is available in very high SA, 22 to 26 mc per mg. In addition, it is counted with greater efficiency than Co<sup>60</sup>B<sub>12</sub>, with a scintillation detector, because it has a much lower

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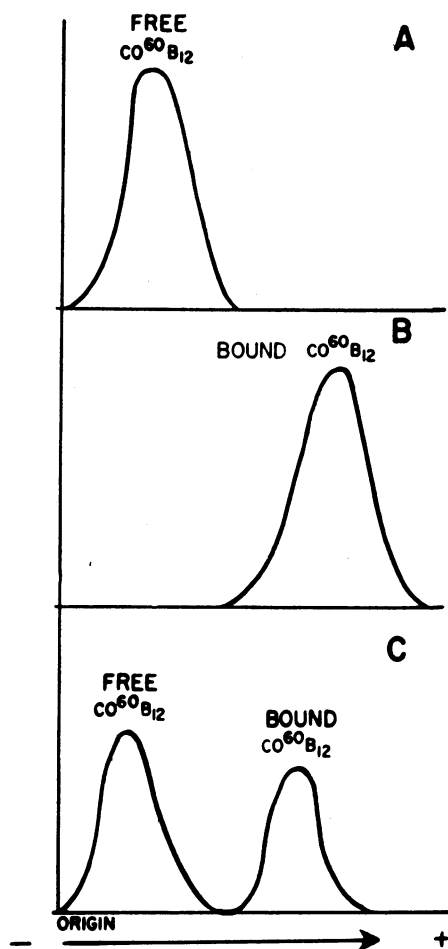


FIG. 1. ELECTROPHORETIC MOBILITY OF FREE AND INTRINSIC FACTOR (IF)-BOUND  $\text{Co}^{60}\text{B}_{12}$ . A.  $\text{Co}^{60}\text{B}_{12}$  alone. B.  $\text{Co}^{60}\text{B}_{12}$  completely bound to IF. C. The same amount of IF incubated with a mixture of  $\text{Co}^{60}\text{B}_{12}$  and unlabeled crystalline  $\text{B}_{12}$ .

energy gamma emission, 0.121 Mev for  $\text{Co}^{57}\text{B}_{12}$  and 1.12 and 1.33 Mev for  $\text{Co}^{60}\text{B}_{12}$ . The radioactivity is detected in a well-type scintillation detector with a 2-inch thallium activated sodium iodide crystal in combination with scaler and gamma-ray spectrometer. The efficiency for  $\text{Co}^{57}\text{B}_{12}$  is approximately  $1.6 \times 10^6$  cpm per  $\mu\text{c}$ .

The only additional special material needed is a potent IF preparation. Several concentrates of hog IF<sup>1</sup> have been used, and apparently the more purified and potent the preparation, the less variable its binding property is in relation to the electrolytes present in the diluent. The more purified concentrates, however, appeared to be less stable when frozen for storage in dilute solution.

The assay procedure may be divided into several important steps. As a guide for the descriptions that follow, these procedures are listed in sequence and each

<sup>1</sup> Kindly supplied by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N. Y.

step is then treated in greater detail: 1) preparation of an IF solution of a concentration such that a constant sample will bind a fixed amount of  $\text{Co}^{57}\text{B}_{12}$  in the absence of unlabeled crystalline  $\text{B}_{12}$ ; 2) separation of the IF- $\text{B}_{12}$  complex from unbound  $\text{B}_{12}$  by precipitation; 3) preparation of a standard curve; and 4) preparation of serum extracts to be assayed.

**Preparation of the IF solution.** The IF is dissolved in an acetate-cyanide-Ringer's (ACR) buffer solution made up of equal volumes of Ringer's solution, aqueous sodium cyanide, 10  $\mu\text{g}$  per ml, 0.17 M acetate buffer, pH 5.6, and distilled water. The IF was more stable when frozen in this diluent than in saline solution. Varying dilutions of this IF stock solution are made with the ACR buffer, and 50- $\mu\text{l}$  samples are incubated with the same quantity of  $\text{Co}^{57}\text{B}_{12}$  to be used in the standard. The amount of IF that binds 60 to 80% of the  $\text{Co}^{57}\text{B}_{12}$  is used in the assay.

**Separation of bound and free  $\text{B}_{12}$ .** The IF- $\text{B}_{12}$  complex is precipitated by the addition of 0.5 ml of 5%  $\text{ZnSO}_4$  followed by 1 ml of 0.175 N  $\text{Ba}(\text{OH})_2$  added dropwise with frequent shaking. To insure the complete precipitation of such a small amount of IF protein, eight  $\mu\text{l}$  of a 25% sterile solution of human serum albumin,<sup>2</sup> 2.0 mg, is added to each tube just before precipitation. Other sources of albumin were found to bind more than 5% of the  $\text{Co}^{57}\text{B}_{12}$  and should not be used. To keep the final solution containing the precipitate below pH 8, the exact ratio of  $\text{Ba}(\text{OH})_2$  to  $\text{ZnSO}_4$  should be checked with phenolphthalein as the indicator.

**Standard curve.** Storage of crystalline  $\text{B}_{12}$  in the very low concentrations used for the standard appears to diminish its stability. Accordingly, serial dilutions of crystalline  $\text{B}_{12}$  are made in the ACR buffer from a concentrated stock solution to give final concentrations of 10, 40, 80, 120, and 160  $\mu\text{g}$  per ml. A fixed amount of  $\text{Co}^{57}\text{B}_{12}$  (60 to 80  $\mu\text{g}$ ) in 50- $\mu\text{l}$  quantities was then added to 1 ml of these standard solutions. The IF is then added (always last), and the mixture incubated at room temperature for 1 hour. It appeared that more consistent results were obtained if the incubation mixture was gently agitated. The precipitation is then carried out as described. Because of the low counting rate, it is desirable to count as much of the supernatant fluid as possible to determine the free  $\text{Co}^{57}\text{B}_{12}$ . An efficient method is to separate the precipitate by passing the mixture through a 14-mm Buchner funnel with suction.<sup>3</sup> At least 2 ml of the filtrate can then be counted (out of the final volume of 2.6 ml). Sufficient counts are recorded for a counting error of 1.2% or less. The percentages of  $\text{Co}^{57}\text{B}_{12}$  bound (B) and free (F) are calculated, and a standard curve is obtained by plotting the B/F ratio as a function of the concentration of unlabeled crystalline  $\text{B}_{12}$ . Replicate determinations vary less than 3% in the percentage bound.

**Preparation of serum extracts.** Because vitamin  $\text{B}_{12}$  in

<sup>2</sup> Albumisol, Merck Sharp & Dohme, Philadelphia, Pa.

<sup>3</sup> For best results use glass-fiber filter paper (type 934-AH, H, Reeve Angel & Co., Inc., Clifton, N. J.).

the blood is bound to a specific carrier protein, it is necessary to extract the serum to be assayed. It is also of vital importance that the extract itself bind none of the Co<sup>57</sup>B<sub>12</sub> to be added subsequently. Several different extraction methods were tested, but some sera failed to be completely deproteinated and substances remaining in the extract would interfere in the assay. The best method proved to be a double boiling procedure in the presence of cyanide, first at pH 4.6 and again at pH 5.6. Table I lists the essential steps. A mixture of equal volumes of serum, distilled water containing sodium cyanide, 10  $\mu$ g per ml, and 0.17 M acetate buffer, pH 4.3, is subjected to boiling in a tightly capped test tube. After 15 minutes 1 vol of 0.08 N NaOH is added, and boiling is resumed for 30 minutes. This procedure will liberate 97 to 100% of 100  $\mu$ g of Co<sup>57</sup>B<sub>12</sub> added to the serum before extraction (as a test for B<sub>12</sub> liberation), and if the sera are fresh, it will remove all of the binding properties of the supernatant fluid. Sera long frozen, or refrozen several times, may yield extracts that appear slightly cloudy and bind 10% to 15% of the Co<sup>57</sup>B<sub>12</sub> to be used in the assay. To remove all traces of these binding substances, the extracts are filtered through a small Seitz filter pad with a syringe and Swinney adapter. Less than 5% of Co<sup>57</sup>B<sub>12</sub> added to the extract is removed by the filter pad, thus indicating no significant loss of free B<sub>12</sub> by this procedure. This extraction results in a final fourfold dilution of the serum. Co<sup>57</sup>B<sub>12</sub> and IF, in the same amounts used for

TABLE I  
*Steps in preparation of serum extract to be assayed for vitamin B<sub>12</sub>*

- |  |
|--|
| A. First boiling, 15 minutes                                     |
| 1 vol serum  |
| 1 vol aqueous sodium cyanide, 10 $\mu$ g/ml                      |
| 1 vol 0.17 M acetate buffer, pH 4.3                              |
| B. Second boiling, 30 minutes                                    |
| 1 vol 0.08 N NaOH  |
| C. Supernatant fluid separated and passed through a Seitz filter |

the standard, are then added to 1 ml of this extract, and the B/F ratio is similarly determined. The B<sub>12</sub> concentration is then obtained by referring to the standard curve. Unknown extracts are routinely assayed in the original 1:4 dilution. If very high levels are anticipated, higher dilutions can be made with the ACR buffer as the diluent.

## RESULTS

*Intrinsic factor solution.* The curve relating the quantity of IF to the percentile binding of that amount of Co<sup>57</sup>B<sub>12</sub> to be used in the assay (60  $\mu$ g) is illustrated in Figure 2 for two different

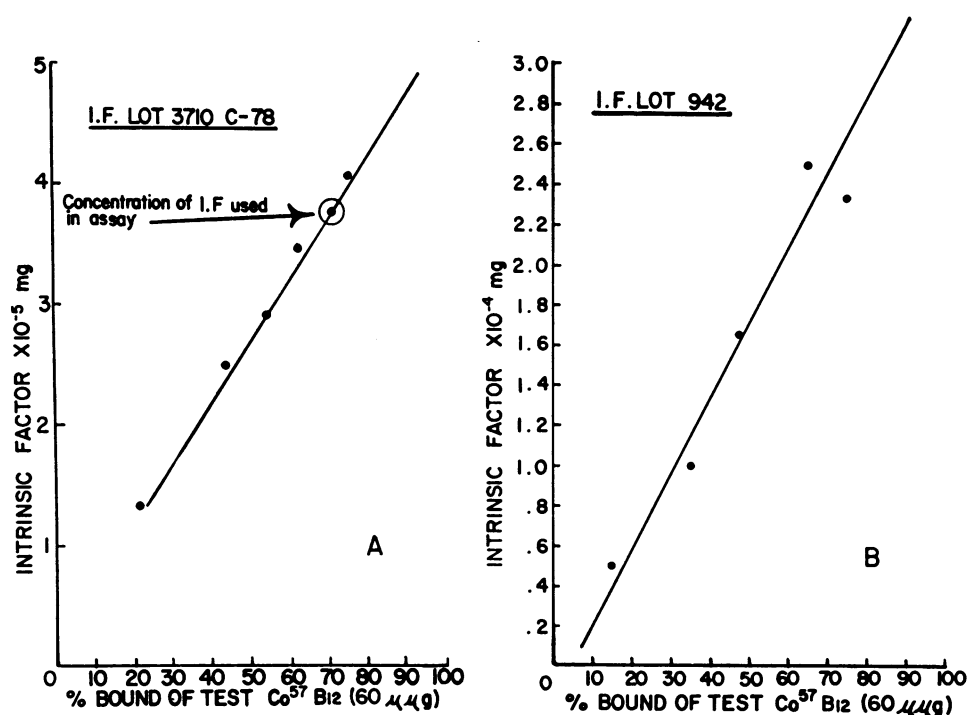


FIG. 2. TWO REPRESENTATIVE IF PREPARATIONS TESTED AT VARYING CONCENTRATIONS FOR THE PERCENTILE BINDING OF THAT AMOUNT OF Co<sup>57</sup>B<sub>12</sub> TO BE USED IN THE ASSAY. The ordinate is the quantity of IF added to a 1-ml vol of acetate-cyanide-Ringer's buffer containing the Co<sup>57</sup>B<sub>12</sub>.

TABLE II

*Recovery of 100  $\mu\text{g}$  of  $\text{Co}^{57}\text{B}_{12}$  after  $\text{ZnSO}_4\text{-Ba}(\text{OH})_2$  precipitation alone, with sterile human serum albumin, and with both albumin and excess intrinsic factor (IF)\**

Substrate	Percentile recovery of $\text{Co}^{57}\text{B}_{12}$
	%
$\text{Co}^{57}\text{B}_{12}$	$99.7 \pm 0.4$
$\text{Co}^{57}\text{B}_{12}$ + albumin	$97.5 \pm 1.1$
$\text{Co}^{57}\text{B}_{12}$ + albumin + excess IF	$< 1.0$

\* Values given are the mean and standard deviation of four representative determinations for each experiment.

IF preparations. For example,  $3.75 \times 10^{-5}$  mg of lot 3710 C-78 will bind 70% of the  $\text{Co}^{57}\text{B}_{12}$ , whereas  $2.4 \times 10^{-4}$  mg of lot 942 was required to bind a similar amount. When frozen for storage, IF appeared to lose some of its binding capacity. As this occurs, the frozen stock solution is diluted in a smaller volume of buffer for the next assay, or a new concentration curve is run. Usually, one can tell from the previous assay how much less to dilute the stock solution. Because of the variable stability of IF in such dilute solutions, a new standard curve is run with each assay.

*Precipitation of IF- $\text{B}_{12}$  complex.* The data summarized in Table II show that the  $\text{ZnSO}_4\text{-Ba}(\text{OH})_2$  precipitation is an effective means of separating free and protein-bound  $\text{B}_{12}$ . All of the 100  $\mu\text{g}$  of the  $\text{Co}^{57}\text{B}_{12}$  is recovered in the supernatant fluid in the absence of protein. If only albumin is present, in a concentration no greater

than 2 mg per ml, 96% to 99% is recovered, whereas with an excess of IF in the mixture, less than 1% of the activity is found in the supernatant fluid.

*Serum extraction.* Table III summarizes the results of both single- and double-precipitation methods in the recovery of  $\text{Co}^{57}\text{B}_{12}$  added to the serum before boiling, as a test of extraction efficiency. The presence of cyanide clearly increases the amount of  $\text{Co}^{57}\text{B}_{12}$  recovered. Sera long frozen, even with double boiling, still retain some binding substances that are subsequently removed by the Seitz filter.

*Standard curve.* Two representative standard curves with sample calculations using different IF preparations are shown in Figure 3. Each point is the mean of duplicate determinations. The sensitivity of the B/F ratio to the increasing concentration of crystalline  $\text{B}_{12}$  is evident by the initial slope of the curve. A different amount of each IF preparation was used for each curve, and this accounts for the different B/F ratios. At low  $\text{B}_{12}$  concentrations, the experimental variation in the B/F ratio will produce less of an error in the determination because of the initial sharp slope of the curve. For most accurate results, therefore, unknown solutions yielding B/F ratios on the more horizontal part of the curve are reassayed in a higher dilution. Likewise, where high levels are expected, as in patients with liver disease or chronic myelogenous leukemia, the initial assay is run at a dilution of 16 times or more.

TABLE III

*Efficiency of various procedures in the extraction of 100  $\mu\text{g}$  of  $\text{Co}^{57}\text{B}_{12}$  from sera and the removal of all  $\text{B}_{12}$ -binding substances from the extracts\**

Extraction	Cyanide	Percentile recovery of 100 $\mu\text{g}$ of $\text{Co}^{57}\text{B}_{12}$ added to serum before extraction	Percentile binding of 100 $\mu\text{g}$ of $\text{Co}^{57}\text{B}_{12}$ by 1 ml of extract†
		%	%
Single boiling, pH 4.6	Absent	$80.0 \pm 3.8$	$14.5 \pm 3.8$
Double boiling, pH 4.6 and then pH 5.6			
Fresh sera	Absent	$87.5 \pm 1.8$	$1.4 \pm 1.6$
Fresh sera	Present	$99.5 \pm 0.9$	
Old frozen sera	Present	$99.0 \pm 1.3$	$12.7 \pm 1.9$
Old frozen sera extract passed through Seitz filter			$< 1.0$

\* Values given are the mean and standard deviation of four representative determinations for each procedure.

†  $\text{Co}^{57}\text{B}_{12}$  is added to extracts containing no radioactivity, incubated for 1 hour, and then precipitated as described.

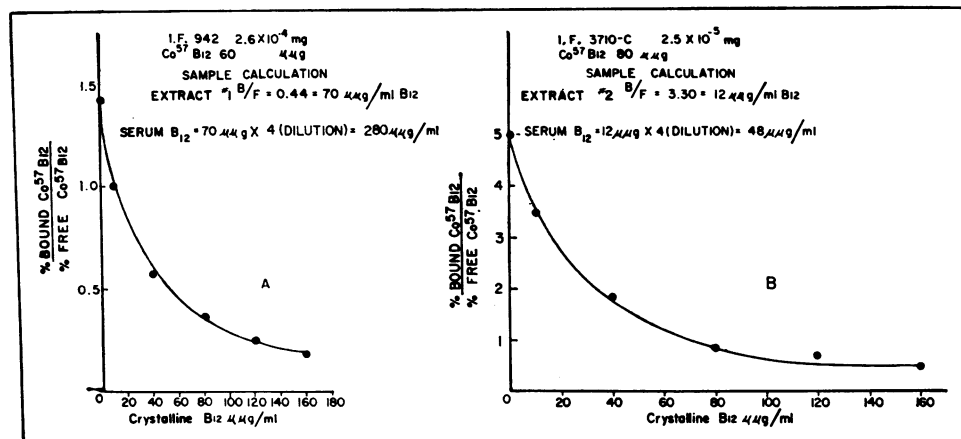


FIG. 3. TWO REPRESENTATIVE STANDARD CURVES WITH SAMPLE CALCULATIONS. Different IF preparations and different amounts of the Co<sup>57</sup>B<sub>12</sub> were used for each curve.

Serum B<sub>12</sub> levels were determined in 20 patients with megaloblastic anemia, 3 patients with chronic myelogenous leukemia, 4 patients with liver disease, and 35 normal control subjects (nurses, medical students, and blood donors). Figure 4 illustrates graphically the serum B<sub>12</sub> level of each individual in each group. Table IV summarizes the range found in each group: 160 to 800 μμg per ml in normal subjects, 4 to 70 μμg per ml in B<sub>12</sub>-deficient patients, 1,000 to 1,970 μμg per ml in the patients with chronic myelogenous leukemia, and 936 to 1,700 μμg per ml in the patients with liver disease.

Recovery of crystalline B<sub>12</sub> added to B<sub>12</sub>-deficient and normal serum before extraction is illustrated in Figure 5. The range of recovery was 85 to 102%, with a mean of 94% for the group. The better recovery from normal serum suggests

TABLE IV

The serum B<sub>12</sub> concentration as determined by radioassay in normal subjects and patients with B<sub>12</sub>-deficient megaloblastic anemia, chronic myelogenous leukemia, and liver disease

Subjects	No.	Range	Mean	±SD
		μμg/ml	μμg/ml	
Normal	35	160– 800	350	143
Megaloblastic anemia	20	4– 70	38	21
Chronic myelogenous leukemia	3	1,000–1,970	1,577	
Liver disease	4	936–1,700	1,425	

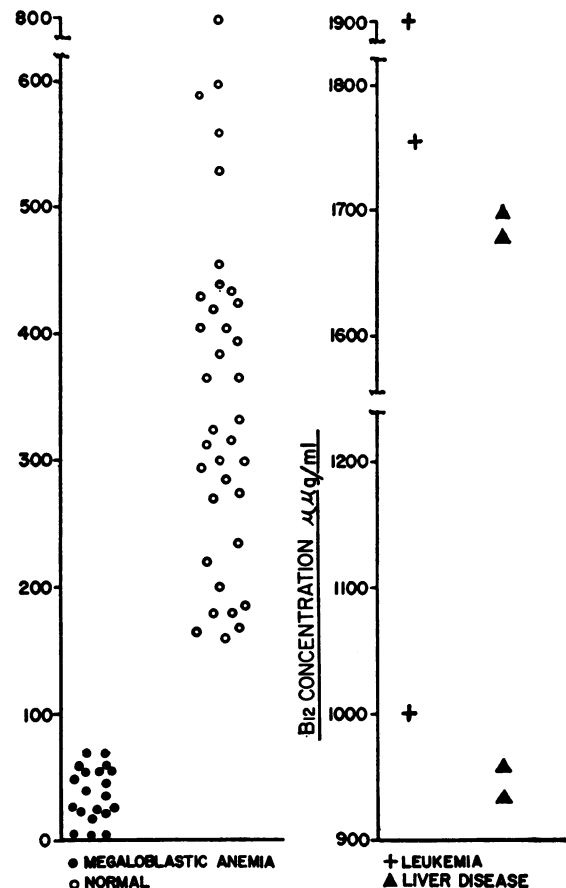


FIG. 4. SERUM B<sub>12</sub> CONCENTRATION IN 35 NORMAL SUBJECTS, 20 PATIENTS WITH B<sub>12</sub>-DEFICIENT MEGALOBlastic ANEMIA, AND PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA AND LIVER DISEASE.

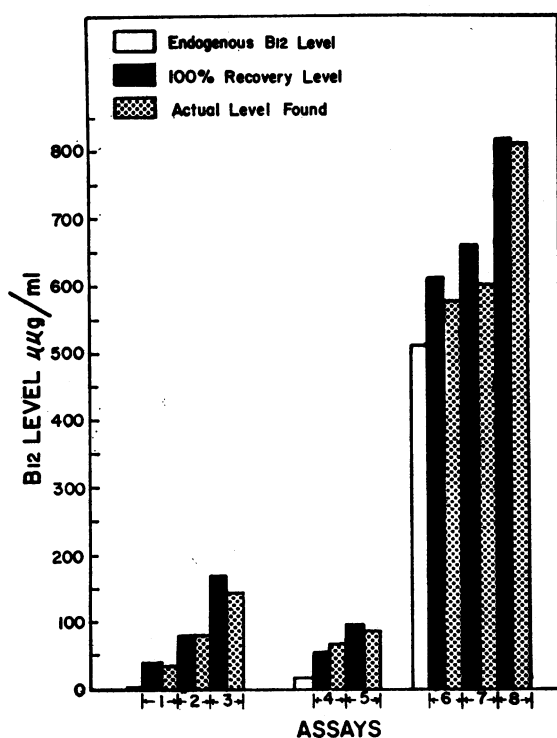


FIG. 5. RECOVERY OF CRYSTALLINE  $B_{12}$  ADDED TO THE SERUM BEFORE EXTRACTION. The range of recovery was 85 to 102%.

that when the specific  $B_{12}$ -binding protein is normally saturated, other serum proteins bind the added  $B_{12}$  with greater tenacity than the specific binder, and there is loss of the  $B_{12}$  during extraction due to coprecipitation with these proteins.

To obtain a more precise comparison of methods, some sera were assayed by this radioassay procedure and microbiologically with *Euglena gracilis* and *Lactobacillus lactis*. These results are summarized in Table V. The *Euglena* assays were consistently and significantly higher, both in normal subjects and patients with  $B_{12}$ -deficient megaloblastic anemia. On the other hand, *Lactobacillus* assays of serum from the  $B_{12}$ -deficient subjects were in closer agreement with the radioisotope assays.

#### DISCUSSION

If radioisotopically labeled molecules are to be used for assay purposes by the principle of competitive inhibition, it is necessary that the reactions between the labeled and unlabeled counterparts be identical. That IF will bind isotopically

labeled  $B_{12}$  and crystalline  $B_{12}$  in an identical way seems assured because the radioactivity is introduced via the cobalt atom, which is an integral part of the  $B_{12}$  molecule. This conclusion is also supported by experimental evidence (6) which demonstrates that  $Co^{60}B_{12}$  and unlabeled vitamin  $B_{12}$  will compete for the binding sites of normal human gastric juice.

Although the instability of IF with prolonged storage is somewhat of a problem, the rapidity of its interaction with vitamin  $B_{12}$  and the slight dissociability of the resulting complex are distinct advantages. Whereas early studies in the course of developing this assay seemed to indicate that the IF- $B_{12}$  complex was dissociable (7), continued investigation into the kinetics of this reaction, for reasons presently under study, suggest that the complex is, in fact, only slightly dissociable. This is in accord with similar findings of Bunge and Schilling (8) using gastric juice as the source of binding. It is this "irreversibility" of the reaction between IF and  $B_{12}$  that enhances the sensitivity of the assay. As the total amount of  $B_{12}$  in the incubating mixture is increased (both labeled and unlabeled), the total amount of  $B_{12}$  bound by

TABLE V

Results of serum  $B_{12}$  assayed by this radioassay method and microbiologically with *Euglena gracilis* and *Lactobacillus lactis*

Subject	Radioisotope assay $\mu\text{g/ml}$	<i>Lactobacillus lactis</i> $\mu\text{g/ml}$	<i>Euglena gracilis</i> $\mu\text{g/ml}$
Normal			
1	278		445
2	278		341
3	270		211
4	400		500
5	318		588
Mean*	308		417
Megaloblastic anemia			
6	16		84
7	26	96	80
8	4†		93
9	4	15	75
10	4		96
Mean	11		85
Megaloblastic anemia			
11	48	24	
12	56	78	
13	70	60	
14	174‡	105	
15	28	36	
Mean	58	59	

\* Mean of matched samples assayed by the different methods.

† Values of 4  $\mu\text{g}$  per ml represent the range 0 to 4  $\mu\text{g}$  per ml.

‡ Subject 14 appeared clinically to be primarily folic acid deficient.

IF remains constant, unlike a system wherein the reactants and the products are in free equilibrium. Hence, a small increment in the quantity of unlabeled B<sub>12</sub> will produce a sharper drop in the B/F ratio of Co<sup>57</sup>B<sub>12</sub>.

A radioassay for vitamin B<sub>12</sub> has been recently reported by Barakat and Ekins (9, 10) applying the same principle of competitive inhibition and using plasma as the binding substance. The assay appeared to be accurate since there was good quantitative recovery of B<sub>12</sub> added to serum. Since the standard curve was a function of millimicrogram levels of B<sub>12</sub>, however, it necessitated the tedious concentration of several milliliters of plasma. In addition, the bound and free radioactive B<sub>12</sub> were separated by time-consuming dialysis. Grossowicz, Sulitzeanu, and Merzbach (11) similarly assayed B<sub>12</sub> using serum as the binder and employing charcoal adsorption to separate bound and free forms. The limit of sensitivity appeared to be 200 to 500  $\mu\mu\text{g}$ , and this also required the concentration of the sample to be assayed. They did not report their findings in patients with vitamin B<sub>12</sub> deficiency, nor recovery of B<sub>12</sub> added to serum.

The results of the comparative assays of the same sera by microbiologic methods must be reviewed with qualified judgement. Unfortunately, the same sera were not assayed by the *Euglena* and *Lactobacillus* organisms because they were carried out at different times over the course of a year. Many factors must be considered in the over-all comparison of results of different methods in different laboratories. Perhaps most important is the standard B<sub>12</sub> solutions. Any variation in the assay of the commercial B<sub>12</sub> used for the standard may be responsible for discrepancies. The higher results obtained with the *Euglena* assay as compared to the *Lactobacillus* assay may be a reflection of the different standard used in each laboratory rather than a difference in the sensitivity of each assay method.

The serum B<sub>12</sub> levels determined by this radioassay correlated well with the expected result, considering the diagnosis of each patient studied. The range found for patients with pernicious anemia, chronic myelogenous leukemia, and liver disease, and for normal control subjects is comparable to those reported by microbiologic assay (12-14).

A question may be raised as to the specificity of this assay for cyanocobalamin, since IF will bind many analogues of this vitamin. Sulfatocobalamin, nitrocobalamin, chlorocobalamin, and some benzimidazole analogues have been shown to compete with cyanocobalamin for the binding sites of IF, although with varying efficiency (7). Whereas none of these compounds are known to be present in the blood, recent reports (15, 16) strongly suggest that hydroxycobalamin may be the physiologic form of cobalamin. By extracting sera in the presence of excess cyanide, however, all cobalamins are converted to the cyano form. Although the final result may be considered a measure of total blood cobalamin, the assay as performed is a direct measure of cyanocobalamin.

This procedure offers several advantages. It is sensitive enough to detect serum vitamin B<sub>12</sub> concentrations as low as 4 to 6  $\mu\mu\text{g}$  per ml. It is rapid, with the extraction and assay completed in a few hours. Isotope-detecting equipment now available in most institutions will meet all the requirements, and technical help can easily acquire the necessary skills. More important, however, is that this is a direct and specific assay for serum B<sub>12</sub> (or cobalamin) not influenced by other products or metabolites that may affect the microbiologic assay (17).

#### SUMMARY

1) A new method of assaying serum vitamin B<sub>12</sub> has been described based on the competitive inhibition of binding of Co<sup>57</sup>B<sub>12</sub> to intrinsic factor.

2) By this method, the serum vitamin B<sub>12</sub> concentration in normal control subjects was 160 to 800  $\mu\mu\text{g}$  per ml, and in patients with B<sub>12</sub>-deficient megaloblastic anemia, 4 to 70  $\mu\mu\text{g}$  per ml. Patients with chronic myelogenous leukemia and liver disease had levels much higher than normal.

3) Crystalline B<sub>12</sub> added *in vitro* to serum before extraction was quantitatively recovered with an efficiency of 85 to 102%.

4) The theoretical and practical considerations of this method as a direct and specific assay for total cobalamin are discussed.

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