A Radioassay for Serum $B_{12}$ Using Unsaturated Transcobalamin I as the $B_{12}$ Binding Protein

By Sheldon P. Rothenberg

In the past several years a number of radioassay procedures for serum vitamin $B_{12}$ have been described. Each method is based on the fact that unlabeled crystalline $B_{12}$ will competitively inhibit isotopically labeled $B_{12}$ (tracer $B_{12}$) from binding to a specific $B_{12}$ binding protein(s). Basic to each procedure is the reaction of $B_{12}$ (labeled and unlabeled) with a limited quantity of binding protein followed then by the separation and quantitation of the free and protein bound $B_{12}$ fractions. By virtue of this competition between the two forms of the vitamin for the binding sites of the protein, the fraction of tracer $B_{12}$ bound is inversely proportional to the concentration of unlabeled $B_{12}$ in the reaction mixture. The first data obtained regarding serum $B_{12}$ concentration with a radioassay system employing intrinsic factor (IF) as the $B_{12}$ binding protein was reported from this laboratory and the protein bound and free $B_{12}$ fractions were separated by precipitation of the IF-$B_{12}$ complex with zinc sulfate and barium hydroxide. In the procedure described by Barakat and Ekins, plasma was used as a source of binding protein, and the free and protein bound $B_{12}$ was separated by equilibrium dialysis. Grossowicz et al. also employed the $B_{12}$ binding proteins of blood, but separated the bound and free $B_{12}$ fractions with charcoal. Lau and co-workers used IF as the binding protein and separated the bound from the free $B_{12}$ by adsorbing the latter on protein coated charcoal. Frenkel et al. more recently described an assay using normal serum as the source of binding protein, and DEAE cellulose for separating the bound and free $B_{12}$.

Although IF would appear to be most suited for use in such a radioassay because of its specific $B_{12}$ binding property, experience in this laboratory with low concentrations of a number of partially purified preparations of this protein has revealed many disadvantages. Since the level of tracer $B_{12}$ generally employed in the radioassay was in the order of 60 pg, the quantity of IF needed to bind 70-80 per cent of this tracer quantity was exceedingly small and such low concentrations of the IF preparations were quite unstable. Consequently, the $B_{12}$ binding capacity of each IF preparation had to be checked just prior to use. Additionally, this variable binding capacity required...
that each time one or more serum extracts were assayed, a new standard
curve had to be determined. Another property of IF which decreased the
sensitivity of the assay was the fact that its B₁₂ binding capacity increased as
the total concentration of B₁₂ in the reacting system increased. This property
of IF will be discussed subsequently.

Other enigmatic properties of some IF* preparations (Wes 942) were also
encountered. One such phenomenon, previously reported from this laboratory¹
was the observation that some IF preparations bound less B₁₂ in distilled
water than they did in extracts of serum. For example, a quantity of IF which
would bind 70 per cent of the tracer B₁₂ in distilled water bound 90 per cent
of the B₁₂ in the extract of a serum from a pernicious anemia patient in re-
lapse. This phenomenon was observed even after the extract had been diluted
50-fold in distilled water. When a more detailed report of this radioassay
appeared,² this enhanced binding capacity of IF in serum extracts appeared
to be corrected by employing a different IF preparation (C-3710) and by
using a Ringer-acetate buffer, similar in composition to the serum extracts,
for determination of the standard curve. However, this enhanced binding ca-
pacity of IF in some serum extracts has again been observed with another
IF preparation (4159 C). Because the standard curve was constructed by
plotting the ratio of percent-bound to percent-free Co⁵⁷B₁₂ (B/F) as a function
unlabeled B₁₂ concentration, the B/F ratio decreased as the unlabeled B₁₂
concentration increased. Since the binding capacity of IF was non-specifically
increased by some serum extracts, an erroneously high B/F ratio was ob-
tained and this resulted in a falsely low estimation of B₁₂ concentration.

Another difficulty encountered with IF has been its variable affinity for
glass, particularly that glass used in disposable test tubes. When these were
substituted in this laboartory for borosilicate glass test tubes, difficulties in
the radioassay were encountered because variable quantities of IF were ad-
sorbed to the surface of the glass.

For these reasons, a systematic search was carried out for a B₁₂ binding
protein which was more stable in dilute solution than IF and which had little
or no change in B₁₂ binding capacity when exposed to variations in hydrogen
ion, electrolyte or B₁₂ concentrations.

Although the nature of the B₁₂ binding proteins in serum have been known
for several years,⁸-¹⁰ two specific B₁₂ binding proteins, transcobalamin I
(TC-I) and transcobalamin II (TC-II) have recently been more specifically
identified by Hall and Finkler.¹¹,¹² Vitamin B₁₂ added to normal serum in vitro
is bound primarily to a beta globulin, TC-II. However, lesser amounts will be
bound to the alpha globulin, TC-I, and some may also be bound by less
distinct proteins. This heterogeneity of B₁₂ binding by normal serum makes it
less than ideal for use in this type of radioassay. However, since the serum
of patients with chronic myelogenous leukemia appears to have a more ho-
mogenous B₁₂ binding protein which has characteristics of the alpha globulin

*IF generously supplied by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River,
New York.
Fig. 1a. (left)—An electrophoretogram obtained after incubating CoB12 with the TC-I serum used in this radioassay (3 ng/ml) and subjecting the mixture to starch gel electrophoresis. The starch was cut in 0.5 cm slices and counted in a well-type scintillation detector. Planimetric analysis of the curves revealed that 2.76 ng/ml of the CoB12 was bound to TC-I and 0.24 ng/ml was bound to TC-II (see text).

Fig. 1b. (right)—An electrophoretogram obtained after incubating CoB12 with normal serum (3 ng/ml) and subjecting mixture to starch gel electrophoresis after removing unbound CoB12 by albumin coated charcoal (see text). The radioactivity in the starch was assayed as described in Fig. 1a. Planimetric analysis of the curves revealed that 1.22 ng/ml of the CoB12 was bound to TC-II and 0.58 ng/ml was bound to TC-I.

TC-I, the use of such a serum for this type of B12 radioassay seemed most ideal. Additionally, studies in this laboratory have indicated that the B12 binding protein of such sera is quite stable under varying pH and electrolyte conditions, and as will be shown subsequently, its binding capacity increases much less than IF or normal serum, when incubated with increasing B12 concentration. This report will describe a radioassay for serum B12 using chronic myelogenous leukemia serum containing unsaturated TC-I as the primary B12 binding protein.

MATERIALS AND METHODS

CoB12 (tracer B12)* with a specific activity ranging from 15–18 μC/μg was used as the isotopically labeled vitamin. Radioactivity was determined in a 2-inch well-type scintillation detector with scaler and gamma ray spectrometer. Sufficient counts were recorded for a counting error of less than 3 per cent.

Serum from a patient with chronic myelogenous leukemia (CML) was used as a source of TC-I. The B12 concentration of samples of this serum taken at different times was within normal limits when assayed by this new method as well as the previous radioassay procedure.2 When tracer B12 was added to this serum in a concentration of 3 ng/ml and sub-

*Supplied through the generosity of Doctor Elmer Alpert, Merck, Sharp and Dohme, West Point, Pa.
ject to starch gel electrophoresis using a borate buffer by the method of Smithies.14 92 per cent of the radioactivity moved with the alpha globulin or TC-I as shown in the electrophoretogram in Fig. 1a. This amount of tracer B12 saturated 94 per cent of the B12 binding capacity of the serum which was calculated to be 3.2 ng/ml. This binding capacity was obtained by determining the per cent of tracer B12 bound by an aliquot of this diluted serum after incubation with 220 pg of B12 (labeled and unlabeled). For comparison, normal serum was similarly incubated with tracer B12 in a concentration of 3 ng/ml and the excess unbound vitamin removed by albumin-coated charcoal as described by Lau and co-workers except that the charcoal was used in a dry form after exposure to the albumin solution. The unsaturated binding capacity of this serum was 1.8 ng/ml. The more heterogeneous binding pattern of this normal serum is shown in Fig. 1b where 68 per cent of the bound tracer B12 now moved with beta globulin mobility (TC-II) and 38 per cent moved with alpha globulin mobility of TC-I. As a screening procedure to find CML serum which will be suitable for use in this radioassay procedure, TC-I can be identified as the primary B12 binding protein by incubating the serum with tracer B12 in a concentration of 1–3 ng/ml and subjecting it to starch gel electrophoresis as described. Some CML serums have a significant amount of TC-II protein which can be detected even when less than saturating amounts of tracer B12 are used for such screening purposes.

This TC-I serum selected for this radioassay was then diluted in varying volumes of 0.9 per cent sodium chloride to find that dilution, of which 50 l would bind 70–80 per cent of 60 pg of Co57B12 used as the tracer B12 in the assay system described below for the standard curve. Once the proper dilution was determined the serum was diluted and stored in small aliquots at −20 C. Freezing and thawing several times did not affect the binding capacity of the diluted serum.

**Standard Curve.** Crystalline B12 was diluted in an acetate-cyanide-Ringer’s (ACR) buffer made up of equal volumes of 0.68M acetate buffer, pH 5.6, distilled water, aqueous sodium cyanide, 10 µg per ml, and Ringer’s solution. For the standard curve 10, 40, 80, and 160 pg of crystalline B12 per ml were made in the ACR buffer. To 1 ml of each B12 concentration, 60 pg of tracer B12 was added in a 50 µl volume. The diluted TC-I serum, in a 50 µl volume, was then added (always last) to each tube and the mixture incubated with gentle agitation for 30 minutes. The protein-bound B12 was separated from the free B12 by precipitation with 0.5 ml. of 5 per cent ZnSO4·7H2O and 1.0 ml. of 0.175N Ba(OH)2 as previously described. An aliquot of the supernatant solution following centrifugation was counted to determine the free Co57B12. From this determination the fraction of Co57B12 bound was calculated and the standard curve obtained by plotting the reciprocal of the fraction-bound (1/FB) as a function of the concentration of unlabeled B12.

**Preparation of Serum Extracts.** The endogenous serum B12 was separated from its protein binders and the serum deprotenated by a method similar to the one previously described except for the use of a higher molarity of acetate buffer. One part serum was mixed with one part 0.68M acetate buffer, pH 4.3, and one part aqueous sodium cyanide, 10 µg per ml. After boiling this mixture for 15 minutes with frequent shaking, one volume of 0.3N NaOH was added and the boiling resumed for an additional 15 minutes. Most extracts were clear with a pH approximating 5.6. Some old sera gave slightly cloudy extracts which did not interfere significantly in the assay. Extracts which were very cloudy were filtered through a small Seitz filter contained in a Swinney adapter. This procedure will remove B12 binding protein fragments without significant loss of free B12. The extraction procedure resulted in a four-fold dilution of the original serum. The same quantity of tracer B12 and TC-I serum as used for determination of the standard curve was then added to 1 ml of the serum extracts, and the 1/FB ratio similarly obtained. The B12 concentration was then determined by reference to the standard curve or by using a simple arithmetic expression derived from the slope of the standard curve.* If very high levels of endogenous B12 were anticipated, the extracts were diluted with ACR buffer prior to assay.

*See appendix for the derivation of the expression used for calculating the B12 concentration from the standard curve shown in Fig. 2.
RESULTS

A typical standard curve and sample calculation illustrated in Fig. 2 shows the linear relationship between the 1/FB ratio and concentration of unlabeled B_{12}. Each point on the curve was the mean of five different assays using the same diluted TC-I serum. For each assay, the unlabeled B_{12} concentrations were freshly prepared from a stock solution of crystalline B_{12} standardized spectrophotometrically (\(E_{1cm}^{1%} = 204\) at 361 m\(\lambda\)m). This standard curve once determined for this lot of TC-I serum was not reassayed for any of the B_{12} recovery experiments or repeat assays of the serum extracts to be described below. However, to insure the constancy of the binding capacity of the stored diluted TC-I serum, with each series of extracts assayed, the per cent tracer B_{12} bound by the TC-I was also determined (initial 1/FB ratio in the absence of unlabeled B_{12}).

Serum B_{12} was measured in patients with B_{12} deficient megaloblastic anemia, normal control subjects, and in some patients with chronic myelogenous leukemia. The results are illustrated in Fig. 3. Normal control subjects had a serum B_{12} concentration ranging from 154 to 672 pg/ml with a mean and S.D. of 375 ± 129. Patients with B_{12} deficient megaloblastic anemia had a B_{12} concentration ranging from 0 to 126 pg/ml with a mean and S.D. of 74 ± 35. Four patients with chronic myelogenous leukemia had serum B_{12} concentrations greater than 1000 pg/ml. These results are comparable to those obtained by microbiological methods using Euglena gracilis\(^{15}\) or Lactobacillus leichmanii\(^{16}\) as the assay organisms. When IF was used as the binding protein in the radioassay procedure,\(^2\) mean serum B_{12} concentration in normal subjects was quite similar being 350 pg/ml. However, the mean for a group of B_{12} defi-
Fig. 3.—The serum B12 concentration in B12 deficient patients, normal control subjects, and four patients with chronic myelogenous leukemia.

* mean and S.D.  • number of subjects in each group
- number of subjects in each group  ▲ number of subjects in each group
† range

Deficiency patients was lower being 38 pg/ml. In retrospect, this lower mean concentration in this group was probably the result of the enhanced binding capacity of IF noted in some extracts of PA serum. As discussed previously, such a phenomenon would tend to give lower B12 values.

The recovery of crystalline B12 added to serum extracts is shown in Table 1. The concentrations of freshly diluted crystalline B12 were made such that the indicated quantities contained in 10 μl volumes were added to 1 ml of the serum extracts. The range of recovery was 84–118 per cent.

The reproducibility of this radioassay was tested by freezing 15 extracts and reassaying them a second time, several weeks later. The per cent variation from the mean of the two determinations is listed as the variance in Table 2. The mean of these variances was 7.4 per cent. Since 13 of the 15 determinations had variances less than 12 per cent, the two with variances of 15 and 20 per cent may have been technical errors.

Although it is not shown in Fig. 2, the standard curve is linear to 200 pg/ml of unlabeled B12. There is a slight increase in slope to 250 pg/ml, and above this concentration the slope increases sharply. Therefore, when the serum B12 level is above 1000 pg/ml, only an approximate B12 value can be calculated using the experimentally obtained 1/FB ratio and the formula derived from the linear portion of the curve. As shown in Table 3 when the fraction bound is 0.12 or greater, the calculated B12 concentration is generally within 25 per cent of the value obtained by diluting and reassaying the extract. When the fraction bound is less than 0.10 (1/FB > 10), the error becomes much more significant. For clinical purposes, however, it is possible to arrive at a reasonable estimate of the B12 concentration if it is not practical or convenient to reassay extracts.
Table 1.—Recovery of crystalline $B_{12}$ from serum extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>$B_{12}$ added (pg)</th>
<th>Expected $B_{12}$ concentration (pg/ml)</th>
<th>Actual $B_{12}$ concentration (pg/ml)</th>
<th>Percent recovery</th>
</tr>
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<tbody>
<tr>
<td>12</td>
<td>10</td>
<td>22</td>
<td>26</td>
<td>118</td>
</tr>
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<tr>
<td>31</td>
<td>80</td>
<td>111</td>
<td>102</td>
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</table>

*Crystalline $B_{12}$ contained in 10 µl volumes was added to 1 ml of extracts.

Table 2.—Reproducibility of $B_{12}$ Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>First $B_{12}$ determination (pg/ml)</th>
<th>Second $B_{12}$ determination (pg/ml)</th>
<th>Percent variance from mean</th>
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<tr>
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<td>309</td>
<td>316</td>
<td>1</td>
</tr>
<tr>
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<td>427</td>
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<td>440</td>
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<td>580</td>
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<tr>
<td>14</td>
<td>68</td>
<td>78</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

Mean = 7.4

*Extracts were kept frozen and re-assayed several weeks later.

Table 3.—Comparison of high serum $B_{12}$ concentrations determined by calculation from the $1/FB$ ratio and by reassaying the extracts at a greater dilution

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction Bound</th>
<th>$1/FB$</th>
<th>Calculated $B_{12}$ concentration (pg/ml)</th>
<th>Reassayed $B_{12}$ concentration (pg/ml)</th>
<th>Percent difference between reassayed and calculated $B_{12}$</th>
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<tr>
<td>1</td>
<td>0.244</td>
<td>4.10</td>
<td>700</td>
<td>743</td>
<td>( +) 6</td>
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<tr>
<td>2</td>
<td>0.160</td>
<td>6.25</td>
<td>1268</td>
<td>1050</td>
<td>( -) 17</td>
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<tr>
<td>3</td>
<td>0.123</td>
<td>8.06</td>
<td>1732</td>
<td>1360</td>
<td>( -) 21</td>
</tr>
<tr>
<td>4</td>
<td>0.080</td>
<td>12.50</td>
<td>5736</td>
<td>3552</td>
<td>( -) 38</td>
</tr>
<tr>
<td>5</td>
<td>0.068</td>
<td>14.70</td>
<td>6862</td>
<td>3520</td>
<td>( -) 49</td>
</tr>
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</table>

**DISCUSSION**

In the past few years, several radioisotopic methods for assaying vitamin $B_{12}$ have been reported. Although all are based on the same principle of isotope dilution, they do vary with respect to methodology. A most ideal radioassay is one which is sensitive, reproducible and not time-consuming.

The use of IF has been abandoned in this laboratory for reasons previously
A RADIOASSAY FOR SERUM \( B_{12} \)

Fig. 4.—The change in binding capacity of IF, a normal serum sample, and the TC-I serum used for this radioassay. The initial binding capacity for each binder at the tracer \( B_{12} \) concentration ranged from 46 to 48 pg. The binding capacity was determined by multiplying the total \( B_{12} \) concentration (tracer and unlabeled \( B_{12} \)) by the per cent of tracer \( B_{12} \) bound.

discussed (vide supra). Lau and associates\(^6\) have reported using IF with satisfactory results, but since these workers use 500 pg of tracer \( B_{12} \), the level of IF employed is also proportionately greater and this probably improves the stability of the protein. It also appears from their data that the \( B_{12} \) binding capacity remains constant at this level of IF since the arithmetic expression derived to calculate the \( B_{12} \) concentration in unknown solutions demands such a fixed capacity. At a tracer \( B_{12} \) level of 60 pg, and a correspondingly smaller quantity of IF to bind 70–80 per cent of this, the \( B_{12} \) binding capacity of IF does not remain constant. The per cent change in \( B_{12} \) binding capacity for IF, normal serum, and TC-I serum, starting at the concentration of tracer \( B_{12} \) and increasing the total \( B_{12} \) concentration by the addition of unlabeled \( B_{12} \) is shown in Fig. 4. The binding capacity of IF had the greatest increase, TC-I increased least, and normal serum had an intermediary increase. Because the binding capacity of TC-I serum increases least of all, a more sensitive radioassay will be obtained by using this binding protein.

The linear plot of the standard curve offers the advantage of deriving a simple arithmetic expression to determine the \( B_{12} \) concentration of unknown extracts after the experimentally derived fraction bound is determined. Although Frenkel and associates\(^7\) derived a linear standard curve by using a semilogarithmic plot of \( B_{12} \) concentration as a function of the percent protein-bound \( B_{12} \), the range of unlabeled \( B_{12} \) employed extended from 50 to 600 pg whereas the range of unlabeled employed in this assay is from 10 to 160 pg.

Since Lau et al.\(^6\) and Frenkel and co-workers\(^7\) both calculate the standards as a function of total \( B_{12} \) (tracer \( B_{12} \) and unlabeled \( B_{12} \)), it is very important to establish accurately the concentration of the tracer \( B_{12} \) used in the assay in order to accurately derive the serum \( B_{12} \) concentration. By plotting the stan-
standard curve against the concentration of unlabeled \( B_{12} \), it is not necessary to know the exact amount of tracer \( B_{12} \) employed. The amount of tracer \( B_{12} \) used can therefore be approximated from the concentration of the stock solution of \( Co^{57}B_{12} \) as determined by the supplier.

Although the radioassay procedures reported have used different methods for separating the bound and free \( B_{12} \) fractions, all apparently produce satisfactory results. Dialysis as employed by Barakat and Ekins\(^3,4\) and Grossowicz et al.\(^5\) is too time-consuming for routine clinical use. The separation of bound and free fractions with DEAE cellulose as recently described by Frenkel and co-workers\(^7\) is less time-consuming than dialysis, but more tedious than either the coated charcoal method of Lau et al.\(^6\) or \( ZnSO_4 \cdot Ba(OH)_2 \) precipitation employed in this laboratory.

Because the acid treatment of serum by the method of Lau et al.\(^6\) to separate \( B_{12} \) from the serum-binding proteins is easier than deproteination of serum, an attempt was made to combine that \( B_{12} \) extraction procedure with this radioassay employing TC-I. Although a reproducible standard curve using TC-I could be obtained at a range of pH from 2 to 7, the \( B_{12} \) levels assayed in PA sera treated at pH 2 or 3 (but not deproteinated) and assayed against a standard curve using TC-I at the same pH, gave higher results than when the extracts were deproteinated at pH 5.6. Since the deproteination extraction method has previously been shown to liberate over 95 per cent of bound \( B_{12},^2 \) the lower \( B_{12} \) levels could not be due to less efficient extraction. The reason for this discrepancy is not yet apparent, but it may be that serum treated at a low pH without removal of the denatured proteins develop some product which may interfere to some extent with the binding of TC-I to tracer \( B_{12} \).

**Summary**

A radioisotopic assay for serum vitamin \( B_{12} \) is described based on the fact the unlabeled \( B_{12} \) will competitively inhibit the binding of \( Co^{57}B_{12} \) to a specific \( B_{12} \) binding protein, transcobalamin I. This protein is stable in dilute solution and at the concentration of \( Co^{57}B_{12} \) used for the assay, its binding capacity increases much less than IF or normal serum as the concentration of \( B_{12} \) increases in the reaction mixture. Because of the stability of the binding capacity of stored frozen TC-I containing serum, only a single standard curve need be established with each lot of binding protein. When a limited quantity of TC-I is incubated with 60 pg of \( Co^{57}B_{12} \) and crystalline \( B_{12} \) concentrations ranging from 10 to 160 pg/ml, a linear standard curve is obtained when the reciprocal of the fraction of \( Co^{57}B_{12} \) bound to TC-I is plotted as a function of unlabeled \( B_{12} \) concentration. This permits use of a simple arithmetic expression to determine the \( B_{12} \) concentration of unknown extracts after experimentally determining the fraction of \( Co^{57}B_{12} \) bound to TC-I. The serum \( B_{12} \) concentration of 25 normal subjects ranged from 154 to 674 pg/ml while the range in 15 \( B_{12} \) deficient patients was 0–126 pg/ml. The \( B_{12} \) concentration in 4 patients with chronic myelogenous leukemia was greater than 1000 pg/ml. The mean variance of 15 serum extracts frozen and reassayed several weeks later was
7.4 per cent. The range of recovery of $B_{12}$ added to serum extracts was 84–118 per cent.

**SUMMARIO IN INTERLINGUA**

Es descriti un metodo de essayage radioisotopic pro le determination del concentrazion seral de vitamina $B_{12}$, utilisante le facto que non-marcate vitamina $B_{12}$ inhibi competitivemente le ligation de $Co^{57}B_{12}$ a transcobalamina I (que es un proteina specificamente ligatori de vitamina $B_{12}$). Iste proteina es stabile in solution a basse concentrazion. Al nivello de concentrazion de $Co^{57}B_{12}$ usate pro le essayage, su capacitate ligatori accresce multo minus que illo de factor intrinsec o que illo de sero normal quando le concentrazion de vitamina $B_{12}$ in le mixtura de reaction es augmentate. A causa del stabilitate del capacitale ligatori de congelate sero a contenuto de transcobalamina I, solo un curva standard debe esser establite pro omne lot de proteina ligatori. Quando un restringite quantitate de transcobalamina I es incubate con 60 pg $Co^{57}B_{12}$ e concentraziones de crystallin vitamina $B_{12}$ de inter 10 e 160 pg per ml, un linea curva standard es obtenite quando le valor recipro del fraction de $Co^{57}B_{12}$ ligate a transcobalamina I es portate graphicamente como function del non-marcate concentrazion de vitamina $B_{12}$. Isto rende possibile le uso de un simple expression arithmetic pro determinar le concentrazion de $B_{12}$ de incognite extractos post le determination experimental del fraction de $Co^{57}B_{12}$ ligate a transcobalamina I. Le concentrazion seral de vitamina $B_{12}$ in 25 subjectos normal variava inter 154 e 674 pg per ml. durante que le correspondente scala in 15 patientes con carentia de vitamina $B_{12}$ se extendeva ab 0 ad 126 pg per ml. Le concentrazion de $B_{12}$ in quatro patientes con chronic leukemia myelogene esseva plus que 1000 pg per ml. Le variantia medie de 15 extractos seral que esseva congelate e re-essayate plure septimanas plus tarde esseva 7,4 pro cento. Le proporcion de retrovate $B_{12}$ post su addition a extractos seral esseva inter 84 e 118 pro cento.

**ACKNOWLEDGMENTS**

I would like to thank Dr. Robert Goldstein for his helpful suggestions in the preparation of this manuscript. I would also like to thank Miss Liisa Huhti for her technical assistance.

**ADDENDUM**

Since submission of this paper the $B_{12}$ concentration of several hundred additional serums has been determined by this radioassy procedure. In no instance did an apparently normal subject have a $B_{12}$ concentration below 200 pg./ml. Since only one of the normal subjects in Figure 3 had a value below this, the lower limit of normal for serum $B_{12}$ by this procedure is probably 200 pg./ml. and not 157 pg./ml.

**APPENDIX**

Derivation of arithmetic expression to calculate $B_{12}$ concentration after experimentally determining the fraction of $Co^{57}B_{12}$ bound to TC-I.

**Equation of a straight line**

$$y = ax + b$$

- $y$ = $y$ coordinate = $1/FB$ ratio
- $x$ = $x$ coordinate = pg/ml unlabeled $B_{12}$
- $b$ = $y$ intercept = 1.3 (for standard curve in Fig. 2)
- $a$ = slope of line = $y - b/x = 0.0158$ (for standard curve in Fig. 2)

$$1/FB = 0.0158 x + 1.3$$

and

$$x = \frac{1/FB - 1.3}{0.0158}$$

and

$$x = (1/FB - 1.3) \cdot (63)$$

and

$$\text{pg } B_{12} \text{ per ml serum} = [63(1/FB) - 82] \cdot (\text{dilution factor})$$
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A Radioassay for Serum B₁₂ Using Unsaturated Transcobalamin I as the B₁₂ Binding Protein

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