A Radioassay for Serum B₁₂ Using Unsaturated Transcobalamin I as the B₁₂ Binding Protein

By Sheldon P. Rothenberg

In the past several years a number of radioassay procedures for serum vitamin B₁₂ have been described. Each method is based on the fact that unlabeled crystalline B₁₂ will competitively inhibit isotopically labeled B₁₂ (tracer B₁₂) from binding to a specific B₁₂ binding protein(s). Basic to each procedure is the reaction of B₁₂ (labeled and unlabeled) with a limited quantity of binding protein followed then by the separation and quantitation of the free and protein bound B₁₂ fractions. By virtue of this competition between the two forms of the vitamin for the binding sites of the protein, the fraction of tracer B₁₂ bound is inversely proportional to the concentration of unlabeled B₁₂ in the reaction mixture. The first data obtained regarding serum B₁₂ concentration with a radioassay system employing intrinsic factor (IF) as the B₁₂ binding protein was reported from this laboratory1,2 and the protein bound and free B₁₂ fractions were separated by precipitation of the IF-B₁₂ complex with zinc sulfate and barium hydroxide. In the procedure described by Barakat and Ekins3,4 plasma was used as a source of binding protein, and the free and protein bound B₁₂ was separated by equilibrium dialysis. Grosso-wicz et al5. also employed the B₁₂ binding proteins of blood, but separated the bound and free B₁₂ fractions with charcoal. Lau and co-workers6 used IF as the binding protein and separated the bound from the free B₁₂ by adsorbing the latter on protein coated charcoal. Frenkel et al7 more recently described an assay using normal serum as the source of binding protein, and DEAE cellulose for separating the bound and free B₁₂.

Although IF would appear to be most suited for use in such a radioassay because of its specific B₁₂ 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 B₁₂ 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 B₁₂ binding capacity of each IF preparation had to be checked just prior to use. Additionally, this variable binding capacity required

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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 laboratory 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 relapse. 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, 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 capacity 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 of 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 obtained 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 laboratory for borosilicate glass test tubes, difficulties in the radioassay were encountered because variable quantities of IF were adsorbed 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, two specific B₁₂ binding proteins, transcobalamin I (TC-I) and transcobalamin II (TC-II) have recently been more specifically identified by Hall and Finkler. 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 homogenous 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 Co\textsuperscript{57}B\textsubscript{12} with the TC-I serum used in this radioassay (3 ng/ml) and subjecting the mixture to starch gel electrophoresis.\textsuperscript{14} 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 Co\textsuperscript{57}B\textsubscript{12} 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 Co\textsuperscript{57}B\textsubscript{12} with normal serum (3 ng/ml) and subjecting mixture to starch gel electrophoresis\textsuperscript{14} after removing unbound co\textsuperscript{57}B\textsubscript{12} 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 Co\textsuperscript{57}B\textsubscript{12} 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 B\textsubscript{12} radioassay seemed most ideal. Additionally, studies in this laboratory have indicated that the B\textsubscript{12} 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 B\textsubscript{12} concentration. This report will describe a radioassay for serum B\textsubscript{12} using chronic myelogenous leukemia serum containing unsaturated TC-I as the primary B\textsubscript{12} binding protein.

**MATERIALS AND METHODS**

Co\textsuperscript{57}B\textsubscript{12} (tracer B\textsubscript{12})\textsuperscript{*} 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 B\textsubscript{12} 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.\textsuperscript{2} When tracer B\textsubscript{12} was added to this serum in a concentration of 3 ng/ml and sub-

\textsuperscript{*}Supplied through the generosity of Doctor Elmer Alpert, Merck, Sharp and Dohme, West Point, Pa.
jected to starch gel electrophoresis using a borate buffer by the method of Smithies. 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 per cent 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 deproteinized 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.
STANDARD CURVE

SAMPLE CALCULATION
TC I bound 38.6% of Co$^{57}$B$_{12}$ in normal extract

$\frac{I/FB}{I/FB_{0.386}} = 2.59$

FROM GRAPH 2.59 = $\frac{1}{B_{12}}$

$B_{12} = (\frac{63}{10.386} - 1) \times$ Dilution factor = 324 pg/ml

OR

$B_{12} = (\frac{63}{2.59} - 1) \times$ Dilution factor

$B_{12} = 324$ pg/ml

Fig. 2.—A typical standard curve obtained by plotting the reciprocal of the fraction of Co$^{57}$B$_{12}$ bound by the TC-I serum as a function of the quantity of unlabeled B$_{12}$. Each point is the mean of five determinations made at different times. The serum B$_{12}$ concentration is obtained by reference to the standard curve or by calculation from the formula derived from the curve as shown in the sample calculation.

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 ($E^{1cm} = 204$ at 361 mp). 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-
A RADIOASSAY FOR SERUM B₁₂

![Graph showing serum B₁₂ concentrations for B₁₂ deficiency, normal subjects, and chronic myelogenous leukemia.]

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
- `†` range

Deficient 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.¹ 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>Endogenous $B_{12}$ (pg/ml)</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>
</thead>
<tbody>
<tr>
<td>12</td>
<td>10</td>
<td>22</td>
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<td>118</td>
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<td>43</td>
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<tr>
<td>31</td>
<td>80</td>
<td>111</td>
<td>102</td>
<td>92</td>
</tr>
</tbody>
</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>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>309</td>
<td>316</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>409</td>
<td>427</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>260</td>
<td>296</td>
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</tr>
<tr>
<td>4</td>
<td>440</td>
<td>409</td>
<td>4</td>
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<tr>
<td>15</td>
<td>60</td>
<td>40</td>
<td>20</td>
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</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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<td>0.244</td>
<td>4.10</td>
<td>700</td>
<td>743</td>
<td>(+) 6</td>
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<td>0.160</td>
<td>6.25</td>
<td>1368</td>
<td>1050</td>
<td>(−) 17</td>
</tr>
<tr>
<td>3</td>
<td>0.123</td>
<td>8.06</td>
<td>1732</td>
<td>1360</td>
<td>(−) 21</td>
</tr>
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<td>4</td>
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<td>3552</td>
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<tr>
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<td>0.068</td>
<td>14.70</td>
<td>6862</td>
<td>3520</td>
<td>(−) 49</td>
</tr>
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**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
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-
dard 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}-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}, 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 B₁₂ added to serum extracts was 84–118 per cent.

SUMMARY IN INTERLINGUA

Es describite un metodo de essayage radioisotopic pro le determination del concentração seral de vitamina B₁₂, utilisante le facto que non-marcate vitamina B₁₂ inhibi competitivemente le ligation de Co⁵⁷B₁₂ a transcobalamina I (que es un proteina specificamente ligatori de vitamina B₁₂). Istè proteina es stabile in solution a basse concentrazione. Al nivello de concentration de Co⁵⁷B₁₂ usate pro le essayage, su capacitate ligatori accresce multo minus que illo de factor intrinsec o que illo de sero normal quando le concentration de vitamina B₁₂ in le mixtura de reaction es augmentate. A causa del stabilitate del capacitate ligatori de congelate sero a contento de transcobalamina I, solo un curva standard debe esser establit pro omne lot de proteina ligatori. Quando un restringite quantitate de transcobalamina I es incubate con 60 pg Co⁵⁷B₁₂ e concentrationes de crystallin vitamina B₁₂ de inter 10 e 160 pg per ml, un linearì curva standard es obtenite quando le valor reciproc del fraction de Co⁵⁷B₁₂ ligate a transcobalamina I es portate graphicamente como function del non-marcate concentration de vitamina B₁₂. Isto rende possibile le uso de un simple expression arithmetic pro determinar le concentration de B₁₂ de incognite extractos post le determination experimental del fraction de Co⁵⁷B₁₂ ligate a transcobalamina I. Le concentration seral de vitamina B₁₂ in 25 subjectos normal variava inter 154 e 674 pg per ml. durante que le correspondentë scala in 15 patients con carentìa de vitamina B₁₂ se extendeva ab 0 ad 126 pg per ml. Le concentration de B₁₂ in quatro patients con chronic leucemia 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 proportion de retrovate B₁₂ post su addition a extractos seral esseva inter 84 e 118 pro cento.

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ADDENDUM

Since submission of this paper the B₁₂ concentration of several hundred additional serums has been determined by this radioassy procedure. In no instance did an apparently normal subject have a B₁₂ 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 B₁₂ by this procedure is probably 200 pg./ml. and not 157 pg./ml.

APPENDIX

Derivation of arithmetic expression to calculate B₁₂ concentration after experimentally determining the fraction of Co⁵⁷B₁₂ bound to TC-I.

equation of a straight line

\[ y = ax + b \]

y = y coordinate = 1/FB ratio
x = x coordinate = pg/ml unlabeled B₁₂
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
pg B₁₂ per ml serum = \[ 63(1/FB) - 82 \] \cdot (dilution factor)
REFERENCES


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