An Isotopic Method for the Determination of Vitamin B₁₂ Levels in Blood

By RUSSEL M. BARAKAT AND ROGER P. EKINS

FOR A PERIOD of about a decade, microbiological assay with Euglena gracilis¹ and other microorganisms² has been accepted as a standard method of vitamin B₁₂ determination in serum and other body fluids. However, this method is time-consuming, entails many sources of error and is not fully reliable. Other methods of assay³ require relatively large quantities of material which preclude their application to the determination of the concentrations found in serum.

With the introduction of technics for labeling the vitamin at high specific activity with radioactive isotopes of cobalt and the reported observations⁴ of specific vitamin B₁₂ binding protein in normal plasma, the basis has been established for the development of an alternative technic possessing sensitivity sufficient for the determination of blood levels of this compound.

The fundamental approach employed by the authors has been that of "saturation analysis."⁷ Recently, this technic has been applied to the assay of serum concentrations of thyroxine,⁶ and, in slightly modified form, to the assay of serum insulin⁸. The assay may be considered to consist of two stages:

1) Extraction of the vitamin from the plasma under investigation (henceforth referred to as the "test" plasma) after heat coagulation of plasma proteins.¹¹,¹²

2) Addition of the extract to a suitable aliquot of a normal plasma (the "standard" plasma) and evaluation of the distribution of vitamin in this plasma between the "bound" (bound to vitamin B₁₂ binding protein) and "free" fractions following separation of the two by equilibrium dialysis.¹³,¹⁴

The value taken by the parameter R₁/₀ (free-to-bound ratio) is dependent upon a number of factors. Of these, the following are relevant to the present context:

a) the concentration of vitamin B₁₂ binding protein present in the standard plasma;
b) the concentration of endogenous vitamin B₁₂ in the standard plasma;
c) the concentration of exogenous vitamin B₁₂ (extracted from "test" plasma);
d) the magnitude of the equilibrium constant governing the binding of the vitamin by its carrier protein.

However, if specimens of the standard plasma are loaded with a range of known amounts of vitamin B₁₂, change in the ratio R₁/₀, will depend solely

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on the only variable—that is, the concentration of exogenous vitamin present in the sample, since all other factors may be held constant.

A curve relating these two parameters can thus be drawn and used to deduce the unknown concentration of B₁₂ extracted from the “test” plasma.

The function of the isotopic label in this form of analysis is thus seen to be twofold:

a) to yield quantitative information regarding the recovery of vitamin B₁₂ through the extraction procedure;

b) to reveal the distribution of the vitamin between the “free” and “bound” fractions after these have been separated.

MATERIALS AND METHODS

Co⁵⁷ vitamin B₁₂ (specific activity 1.7 µc. per µg.) was obtained from Abbott Laboratories, diluted in physiologic saline to a concentration of 0.05 µg. per ml. and stored at −20 C. When required for use, small batches of this stock solution were further diluted to a working concentration of 0.01 µg. per ml. and retained under similar conditions.

Standard plasma was obtained from pooled bank blood and kept at 4 C. Actate buffer (pH 4.5, 0.3 M) used in the assay was made up containing a small supplement of KCN added at the rate of 40 mg./L. of buffer.

0.1 ml. of the vitamin B₁₂ working solution (containing 1 mµg.) was diluted with 1 ml. acetate buffer and counted in a well-type scintillation counter. 1–5 ml. of fresh or frozen plasma were then added and the mixture allowed to equilibrate for about 20 minutes. (The pH of the plasma/buffer mixture usually fell to between 5 and 6). The vitamin was subsequently released from binding protein in the plasma by heating the mixture in a boiling water bath for 30 minutes followed by separation of the coagulated proteins by centrifugation. The protein precipitate was washed once with 3 ml. of a diluted buffer solution (0.5 ml. buffer and 2.5 ml. distilled water), the washing and initial supernatant then combined, and taken down to approximately 1 ml. under reduced pressure. Recovery of B₁₂ was estimated at this stage by counting the extract in the well-scintillation counter. 0.1 ml. of standard plasma was finally added to the concentrated extract and the mixture allowed to equilibrate for 2 hours at room temperature.

Standard vitamin B₁₂ plasma dilutions were made up by addition of 0.5-ml. quantities of the standard plasma to aliquots of working solution containing vitamin B₁₂ ranging from 0.5 to 5.0 µtg. These standard solutions were likewise allowed to equilibrate for 2 hours at room temperature.

After equilibration, each specimen was made up to 3 ml. with saline and transferred to dialysis bags cut from 18/32” Visking dialysis tubing. The length of each bag was kept roughly constant at about 15 cm. The entire group of plasma dilutions was then dialyzed for 48 hours, each against 300 ml. physiologic saline; the bags were finally thoroughly rinsed under tap water, transferred to graduated counting tubes, pierced, and the volume of the contents of each tube made up to 5 ml. The residual activity in the bags was finally estimated in the scintillation counter, the usual corrections being applied for background and sample volume.¹⁵

All radioassays were taken to at least 10⁴ counts and counting errors were never greater than 3 per cent.

Microbiologic assay of plasma specimens was performed using Lactobacillus leichmanii.¹¹

Calculation

Distribution of vitamin B₁₂ between bound and free fractions may be quantitatively expressed in a number of ways. In this study, the ratio R / is chosen as the descriptive parameter and calculated by use of the expression:

\[ R = \frac{[F]}{[B]} \]
Fig. 1.—A typical standard curve. The ratio of free to bound \( R_{f/b} \) plotted against concentration \( Co^{57}B_{12} \) in plasma.

\[
R_{f/b} = \frac{Cr - Cb}{Cb}
\]

where \( Cb \) = counts retained in the bag and

\( Cr \) = total counts initially placed in the bag.

The experimentally derived values for \( R_{f/b} \) were plotted against concentration of exogenous vitamin \( B_{12} \) (fig. 1). The concentration of vitamin in the unknown plasma specimen could thus be derived from this curve by interpolation of the corresponding value of \( R_{f/b} \).

Corrections for extraction losses and the amount of vitamin initially added to the test plasma were made and the concentration of endogenous material present in the latter derived using the formula:

Total amount of vitamin \( B_{12} \) in the unknown specimen =

\[
\frac{Ca \times B}{Cr} - W \frac{V}{(\text{mug./ml.})}
\]
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Table 1.—Results of Repeat Assays on Five Pooled Bank Plasmas

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Vitamin Concentration (μg./ml.)</th>
<th>Mean ±</th>
<th>S.D.*</th>
<th>Vitamin Concentration</th>
<th>Microbiological Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>420</td>
<td>440</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>150</td>
<td>148</td>
<td>7</td>
<td>190</td>
<td>200</td>
</tr>
<tr>
<td>III</td>
<td>450</td>
<td>420</td>
<td>23 (5.6%)†</td>
<td>608</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>390</td>
<td>404</td>
<td>20</td>
<td>750</td>
<td>526</td>
</tr>
<tr>
<td>V</td>
<td>322</td>
<td>310</td>
<td>18 (5.7%)†</td>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>

*Standard deviation (16).
†Coefficient of variation from the mean = \( \frac{S.D. \times 100}{\text{mean}} \).

or after substituting \( \frac{Ca}{S} \) for \( W \) and rearranging,

\[
= \frac{Ca}{V} \left( \frac{B}{Cr} - \frac{1}{S} \right)
\]

where
- \( Ca \) = activity added to test plasma (cpm).
- \( V \) = volume of test plasma (ml.).
- \( B \) = amount of vitamin in unknown specimen deduced from calibration curve (μg.).
- \( W \) = weight of \( B₁₂ \) initially added (μg.).
- \( Cr \) = activity recovered after extraction (cpm).
- \( S \) = specific activity of labeled vitamin (cpm per μg.).
Fig. 2.—Recovery of added vitamin. The total amount assayed is plotted against amount added.

RESULTS

Table 1 shows the results of assays, repeated on separate occasions, of samples of plasma obtained from pooled bank blood (diluted to an extent of about 30 per cent with anticoagulants). The coefficient of variation from the mean in each of the larger series is about 5–6 per cent.

Figure 2 represents the results of assays on two plasmas in which endogenous vitamin was augmented by known increments of exogenous unlabeled material. Consequent increases in vitamin concentration were accurately reflected in the experimental results.

Figure 3 illustrates an experiment in which assays were carried out on a range of volumes of the same pooled plasma. An identical amount (0.5 mg. in this series) of labeled vitamin was added to each specimen at the first stage of the assay in the usual way. The expected proportionality between total endogenous vitamin content and initial specimen volume is seen to obtain.

In table 2, the results of assays on venous blood obtained from young male
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Fig. 3.—Assay of a range of volumes of the same plasma specimen.

and female laboratory personnel are presented. The range of values obtained in 39 subjects was from 330 to 1070 $\mu$g./ml. with an average of 611 ± 167 $\mu$g./ml. These values compare with a range of from 270–1270 $\mu$g./ml. with a mean of 640 ± 100, which was obtained by other workers using a microbiological technic. The table also shows values obtained in various pathologic conditions. Four patients with clinical pernicious anemia were found to yield concentrations of from 11 to 140 $\mu$g./ml. A small group of vegetarians displayed values below 300 $\mu$g.—that is, below the tentative normal range established using the present technic.

DISCUSSION

The technic described possesses the merit of conceptual and technical simplicity; moreover, the results obtained have consistently displayed a gratifying degree of reproducibility when specimens have been repeatedly assayed on different occasions. Nevertheless, potential sources of error which have not been investigated in this study remain in the technic and may in the future be shown to demand modification of the method as described. The method depends upon the assumption that free vitamin $B_{12}$ is liberated from plasma by heat, but it is possible that a fraction of the released material is present as small dialyzable peptide complexes which may not have binding properties identical to the free vitamin.

Other points of weakness are a) possible incorrect estimation of recovery of vitamin $B_{12}$ from the plasma under investigation, as revealed by the exogenous labeled material, and b) artifactual displacement of the vitamin from bound to free fractions by other compounds present in the plasma extract. An error of the first type would arise if all or some part of the vitamin $B_{12}$ present in the "test" plasma equilibrated only slowly with exogenous labeled vitamin and if the extraction procedure succeeded in removing only a small
Table 2.—Serum Levels in Normals and in Different Disease States

<table>
<thead>
<tr>
<th>Disease State</th>
<th>No. of Subjects</th>
<th>Serum No.</th>
<th>Vitamin Concentration (μg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>39</td>
<td>–</td>
<td>330–1080*</td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>4</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>74</td>
</tr>
<tr>
<td>Vegetarians</td>
<td>5</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>125</td>
</tr>
<tr>
<td>Folic acid deficiency</td>
<td>1</td>
<td></td>
<td>257</td>
</tr>
<tr>
<td>Postgastrectomy state</td>
<td>2</td>
<td></td>
<td>248</td>
</tr>
<tr>
<td>Steatorrhea</td>
<td>3</td>
<td></td>
<td>400</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td>1</td>
<td></td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>7000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>9900</td>
</tr>
<tr>
<td>Chronic lymphatic leukemia</td>
<td>580</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic lymphatic leukemia</td>
<td>4410</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with renal involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* (mean = 611)
(S.D. = 167).

fraction of the total vitamin. Under these circumstances, the specific activity of the extracted vitamin would exceed that of residual material and measurement based on radioactive assays would not represent recovery of the endogenous compound. This possibility is difficult to test experimentally; however, the presence of a substantial pool of vitamin B12 in plasma which is not extractable under the conditions used is not a likely eventuality.

In the present study, recovery of radioactive vitamin added at the first stage of the assay has usually fallen in the vicinity of 80 per cent. Further washing of the protein precipitate resulted in an increase in this value until virtually all radioactivity was extracted. However, protracted washing of the protein residue was not considered necessary in view of the probability that recovery of at least the portion of endogenous vitamin released by heating is indicated by recovery of added radioactivity.

Labeled vitamin B12 administered to a rat in vivo was extractable from the plasma up to at least 24 hours following administration, to an extent identical to that of vitamin added to the same plasma in vitro. Nevertheless, fully conclusive experimental evidence that exogenous labeled vitamin B12 added to plasma in vitro functions as a tracer for the entire endogenous vitamin moiety in the extraction procedure was not obtained by the authors, and the possibility remains that an underestimate of the amount of B12 present is made. However, the same criticism may validly be applied to any method involving extraction, the more so when no radioactive tracer material is employed to indicate losses.

Binding by vitamin B12 proteins of compounds other than vitamin B12 has
not been reported. Were such compounds to exist in the plasma extract, they might simulate vitamin B₁₂ in the dialysis system and result in elevated estimates of the amount of vitamin present in the extract. Experience with thyroxine binding protein would suggest that this is improbable, but the possibility of competitive binding of this kind for the protein binding sites remains.

A further possibility of error in this technic would arise if substantial amounts of the binding protein were carried over in the extraction procedure. This would vitiate direct comparison of distribution ratios obtained with extracted vitamin on the one hand and unextracted standards on the other. This possibility was tested by dialysis of extracted vitamin B₁₂ in the absence of added standard plasma. Distributions approximating those obtained with free B₁₂ were obtained, however, suggesting that extracted and unextracted B₁₂ may legitimately be compared in the dialysis system.

The effect of carry over of endogenous B₁₂ BP would be to reduce the estimated concentration of B₁₂, and for this reason the values for myelogenous leukemic plasmas containing greatly elevated concentrations of binding protein might be considerably depressed. Nevertheless, the values observed for such sera were such as not to present any clinical ambiguity.

About 5–10 per cent of the added vitamin appeared to be adsorbed to the walls of the dialysis bags. Although this effect alters the shape of the standard calibration curve, particularly at high concentrations of “free” vitamin, it does not in principle introduce an error into the assay. Likewise, the time occupied in dialysis is not critical at the concentrations of vitamin used provided that it is constant for any given group of unknowns and standards assayed together. Most of the vitamin is dialyzed out in 24 hours. Nevertheless, it was felt preferable to dialyze the entire free B₁₂ moiety out of the bags which necessitated a dialysis time of 48 hours. No significant alteration in results was observed when the dialysis was conducted at 4°C.

Estimates of $R^f/s$ are most accurate when this takes a value close to unity. For this reason, it was considered advisable to select a working ratio of test to standard plasmas to yield $R^f/s$ values of this order; but where abnormally high or low vitamin B₁₂ concentrations were suspected, appropriately smaller or larger volumes of the test plasma were taken.

The sensitivity of this technic is largely dependent on the specific activity of vitamin B₁₂ available. In this study, adequate count rates were obtained by adding 1.0 mμg. labeled compound to the sample of plasma taken for investigation, and amounts of endogenous material down to approximately 20 μg./ml. (using 5 ml. plasma) could be assayed, though with a poor degree of reliability. It should be stressed that if the amount of vitamin B₁₂ being assayed is considerably less than the amount of exogenous radioactive material, the accuracy of this determination may considerably decrease. However, use of labeled material approximately ten times the specific activity of that available to the authors can be prepared, and considerably enhanced sensitivities are in principle attainable by the technic.

**SUMMARY**

Vitamin B₁₂ is normally present in plasma mainly bound to a specific binding protein. Addition of exogenous vitamin ultimately results in saturation of
protein binding sites and excess vitamin remains in free form. The ratio of free to bound fractions thus quantitatively depends upon the concentration of exogenous compound. This observation has been utilized to determine amounts of vitamin B₁₂ extracted from the blood of normal subjects and of patients with certain pathologic conditions.

The method is simple and reproducible. The sensitivity of the method is such that vitamin levels down to roughly 20 μg/ml may be evaluated using labeled vitamin B₁₂ of a specific activity of about 1 μc./μg. Repeated assays on identical specimens of normal plasma have shown a reproducibility of about 5–6 per cent. Results on 39 normal subjects gave a range of 330–1070 μg/ml with an average of 611 ± 167. Values observed in plasma taken from patients suffering from pernicious anemia were around 100 μg/ml or less. Results on subjects with other pathologic conditions are also presented and the limitations of the method are discussed.

**Summario in Interlingua**

Vitamina B₁₂ es normalmente presente in le plasma, primarimente in un forma ligate a un specific proteina ligatori. Le addition de vitamina B₁₂ exogene resulta ultimemente in le saturation del sitos de ligation del proteina, e un excesso de vitamina remane in forma libere. Per consequente le proportion del libere al ligate fraction depende quantitativamente del concentration del composito exogene. Iste observation ha essite utilisate pro determinar Ic quantitates de vitamina B₁₂ extrahite ab le sanguine de subjectos normal e de patientes con certe conditiones pathologic.

Le methodo es simple e reproducibile. Le sensibilitate del methodo es tal que nivellos de vitamina usque a non plus que circa 20 μg per ml pote esser evaluata con le uso de marcate vitamina B₁₂ de un activitate specific de circa 1 μc per μg. Repetite essayos con specimens identic de plasma normal ha monstrate un reprodicibilitate de circa 5 a 6 pro cento. Le resultatos obtenite ab 39 subjectos normal monstrava un distribution ab 330 ad 1070 μg per ml, con un valor medie de 611 ± 167. Valores observate in plasma obtenite ab patientes con anemia perniciose esseva circum 100 μg per ml o minus. Le resultatos in subjectos con altere conditiones pathologic es etiam presentate. Le limitates del methodo es signalate.

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**REFERENCES**

AN ISOTOPIC METHOD FOR DETERMINING B12 LEVELS

3. Ekins, R. P.: To be published.

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