Measurement of Serum Vitamin B₁₂ Level Using Radioisotope Dilution and Coated Charcoal

By Kam-Seng Lau, Chester Gottlieb, Louis R. Wasserman and Victor Herbert

In previous reports, it was shown that uncoated charcoal adsorbs both free vitamin B₁₂ and B₁₂ bound to proteins, but charcoal precoated by mixing with saturating quantities of albumin adsorbs only free B₁₂. This finding has been confirmed. The charcoal particles may be considered as solid microsponges and the albumin coat as a molecular sieve surrounding each sponge. Small molecules such as B₁₂ pass through the coat and continue to be adsorbed, but large molecules such as proteins are excluded from adsorption. This provides a system akin to, but much more rapid than dialysis for the separation of free from bound B₁₂. This property of coated charcoal has been used in assays for: (1) intrinsic factor (IF); (2) unsaturated B₁₂ binding capacity of gastric juice; (3) antibody to IF; (4) unsaturated B₁₂ binding capacity of serum.

These assays suggested the use of coated charcoal for measuring serum B₁₂ levels. The principle of radioisotope dilution is also applied, by using the unknown quantity of nonradioactive B₁₂ released from serum to dilute the specific activity of a known quantity of Co⁵⁷B₁₂. A solution of intrinsic factor concentrate (IFC) with a B₁₂ binding capacity less than the quantity of added Co⁵⁷B₁₂ is used to bind a portion of the mixture of radioactive and nonradioactive B₁₂—i.e., to “biopsy” the pool of B₁₂. The B₁₂ not bound to IFC is removed by addition of coated charcoal. This series of events is depicted in figure 1. The quantity of radioactive B₁₂ bound to IFC is compared with the IFC control, and the B₁₂ level of the unknown serum is then obtained from a simple equation.

Methods

Albumin-Coated Charcoal

This is prepared by mixing equal volumes of a 5 per cent aqueous suspension of Norit “A” pharmaceutical grade charcoal* and a 1 per cent aqueous solution of bovine serum albumin. Optionally, excess albumin may be removed by replacing the supernatant with fresh water. This mixture is stored at 5 C.; it has been stored for up to a month without loss of usefulness. Alternatively, the mixture may be lyophilized and stored at room temperature indefinitely.

*Norit “A” charcoal is obtained from Amend Drug and Chemical Co., Inc., New York, N. Y.

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Fig. 1.—Schematic representation of serum vitamin B₁₂ assay using radioisotope dilution and albumin-coated charcoal.

Other large molecules such as fibrinogen, globulins, dextrans, Ficoll (Pharmacia, Inc.), and hemoglobin can be used in place of albumin to coat charcoal in the separation of free from bound B₁₂. For serum B₁₂ assay we have found that hemoglobin solution used in the proportion of 1 part hemoglobin solution to 20 parts charcoal by weight (or 1 part hemoglobin powder to 10 parts charcoal by weight), will substitute for albumin-coated charcoal and has the advantage of giving consistently lower supernatant controls. Albumin-coated charcoal occasionally gives high supernatant controls in the order of 5 per cent of added Co³⁺ B₁₂. This does not occur with hemoglobin-coated charcoal where the supernatant controls are usually well below 1 per cent. Hemoglobin solutions can be prepared from washed red cells as described for hemoglobin electrophoresis, or purchased commercially as hemoglobin powder which is cheaper than albumin. The vitamin B₁₂ in red cells is bound and does not interfere with the assay.

The hemoglobin is prepared as follows: discarded human red cells are thrice washed with 0.9 per cent saline, then hemolyzed with an equal volume of distilled water, followed by addition of one half volume of toluene. The mixture is shaken vigorously for 5 minutes, then centrifuged for 15 minutes at 3000 rpm. The top two layers, consisting of toluene and cell debris, are discarded, and the bottom layer, consisting of hemoglobin, is passed through Whatman #1 filter paper to yield a clear red filtrate. The hemoglobin content of the filtrate is determined in conventional manner, and the hemoglobin concentration is adjusted with distilled water to 10 g. per 100 ml.

National Formulary Intrinsic Factor (NFIF)*

A stock solution of 100 mg. NFIF in 100 ml. 0.9 per cent saline is prepared. One ml. of this stock solution is made up to 100 ml. with saline to provide a working solution of 10 µg. NFIF per ml. This solution has a B₁₂ binding capacity of approximately 800 pg. per ml. The stock NFIF solution is stored at −20 C. It may be stored for more than a year without loss of usefulness. The working NFIF solution may be stored between usage frozen or at 5 C. There may be a slight drop in the B₁₂ binding capacity with such storage but this does not affect the assay since the crucial point is not the use of exactly 10 µg. of NFIF but use of that quantity of NFIF which will bind approximately 60 per cent to 80 per cent of the added Co³⁺ B₁₂. The B₁₂ binding capacity of the NFIF solution is determined as previously described, and the proper quantity of NFIF for use in the assay is then known.

*Obtained from the National Formulary, American Pharmaceutical Association, Washington, D. C. (Price: $5. per Gm). One test unit of NFIF in the Schilling test is 50 mg.
Table 1.—Serum B₁₂ Assay Protocol

<table>
<thead>
<tr>
<th>Sample</th>
<th>0.5 Per Cent Saline</th>
<th>Unknown Serum</th>
<th>0.25N HCl</th>
<th>1000 pg./ml. Co⁵⁷B₁₂</th>
<th>10 µg./ml. Std. NFIF*</th>
<th>0.9 Per Cent Saline</th>
<th>Albumin-Coated Charcoal</th>
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<td>1.5</td>
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</table>

*National formulary intrinsic factor.

†The Co⁵⁷B₁₂ binding capacity of 5 µg. NFIF is derived from this sample.

†The supernatant controls contain less than 1 per cent of the added radioactivity. The counts per minute of the supernatant controls are subtracted from those of the unknown serum and NFIF control respectively to obtain net counts.

In those areas of the world where NFIF is difficult to obtain, depepsinized pooled normal human gastric juice can be used.

Vitamin B₁₂ (Cyanocobalamin) Standard

Cyanocobalamin Standard, U.S.P., is diluted to 1000 pg. per ml. in distilled water.

Co⁵⁷ Vitamin B₁₂

The Co⁵⁷ B₁₂* has a specific activity of approximately 11 µc. per µg. This is diluted with distilled water to give 1000 pg. Co⁵⁷B₁₂ per ml., and can be stored at 5 C. for up to 3 months without loss of usefulness.

Procedure for Standardization of Co⁵⁷B₁₂

Every new lot of Co⁵⁷B₁₂ (1000 pg./ml.) prepared for use in the assay is standardized by reverse isotope dilution against the B₁₂ standard (1000 pg./ml.). Five tenths ml. of Co⁵⁷B₁₂ added to 0.5 ml. of cold B₁₂ should result in a fall of exactly 50 per cent in the Co⁵⁷B₁₂ binding capacity of the NFIF. If the Co⁵⁷B₁₂ contains more than 1000 pg. per ml., the fall will be less than 50 per cent; if it contains less than 1000 pg. per ml., the fall in binding capacity will be greater than 50 per cent. The actual quantity of Co⁵⁷B₁₂ present can be calculated for any percentage change in the Co⁵⁷B₁₂ binding capacity of the NFIF by using the following equation:

\[ \text{pg. Co}^{57}\text{B}_{12} = \frac{\text{pg. Cold B}_{12}}{\frac{B'}{B - B'}} \]

Where \( B = \text{Net cpm of tube containing NFIF and Co}^{57}\text{B}_{12} \)

\( B' = \text{Net cpm of tube containing NFIF, Co}^{57}\text{B}_{12} \text{ and standard cold B}_{12} \)

This equation is derived from equation (5) shown under calculation of serum B₁₂ level.

Assay Procedure

Table 1 summarizes the assay procedure. All tests are done in duplicate in 10-ml. test
MEASUREMENT OF SERUM VITAMIN B₁₂ LEVEL

One-half ml. of unknown serum is added to 1.5 ml. 0.9 per cent saline in the tubes for unknown serum and serum supernatant control. The tubes for NFIF control and NFIF supernatant control contain 2.0 ml. saline. These are run in parallel to correct for volume changes caused by heating. To each tube 0.5 ml. 0.25 N hydrochloric acid is added. The tubes are capped with cotton wool, heated in a boiling water-bath for 15 minutes, and then cooled with tap water. Five tenths ml. Co⁵⁷B₁₂ is added to each tube and the contents are mixed for 10 seconds. Five tenths ml. NFIF is added to the unknown serum and NFIF control, and equal volume of saline is added to the supernatant control. The contents are mixed before and again after adding 2 ml. of coated charcoal. The tubes are centrifuged at 3000 rpm for 15 minutes and the supernatant fluid is decanted into counting tubes. The radioactivity in the supernatant fluid is counted in a well-type scintillation counter.†

**Calculation of Serum B₁₂ Level**

The counts per minute (cpm) for the supernatant controls are subtracted from those for the unknown and the NFIF control respectively, in order to obtain net counts. The serum B₁₂ level is calculated from the following formula:

\[
\text{pg. } B₁₂ \text{ per ml. serum} = 2 \times \frac{B}{B'} - 1
\]

Where \( B \) = Net cpm of NFIF control tube
\( B' \) = Net cpm of tube with unknown serum.

**Derivation of Formula**

Let \( M \) = mass of Co⁵⁷B₁₂ added and \( R \) its radioactivity (cpm).
Let \( m \) = mass of Co⁵⁷B₁₂ bound by NFIF and \( B \) its radioactivity (cpm).

Specific activity of Co⁵⁷B₁₂ = \( \frac{R}{M} = \frac{B}{m} \) .......................... (1)

\[
R = \frac{B \times M}{m}
\] .......................... (2)

Let \( B' \) = radioactivity (cpm) of Co⁵⁷B₁₂ bound by NFIF after radiodilution of \( M \) by a mass of \( m' \) cold B₁₂.

New specific activity after radiodilution = \( \frac{R}{M + m'} = \frac{B'}{m} \) ........(3)

Substituting for \( R \) from equation (2)

\[
\frac{B \times M}{m} = \frac{B'}{m'} \frac{m}{M + m'}
\] .......................... (4)

\[
\frac{B \times M}{m} = \frac{B' \times (M + m')}{m}
\]

All glassware must be scrupulously clean, since contamination with B₁₂ voids the assay. Vacutainers #3200 (Becton, Dickinson & Co., Rutherford, N. J.) are free from traces of vitamin B₁₂, and may be used. Plasma may be used, provided it is not heparinized plasma. Heparin binds B₁₂, resists destruction by heat and acid, and attaches to serum protein; this combination of characteristics will cause the charcoal assay to yield false high results for serum B₁₂ binding capacity and false low results for serum B₁₂ level.

†The same protocol has been successfully used for assay of erythrocyte B₁₂ levels. Five tenths ml. of packed washed red cells is used in place of serum and deionized water in place of physiologic saline. Debris from the hemolysed cells does not interfere with the assay, and is brought down together with the charcoal during centrifugation. By charcoal assay, normal erythrocyte B₁₂ levels are approximately 25 per cent of serum levels.
Fig. 2.—Recovery of known quantities of crystalline B₁₂ added to a B₁₂-deficient serum.

\[
\begin{align*}
B & \times M = B'(M + m') \\
\frac{B}{B'} & \times M = M + m' \\
m' & = \frac{B}{B'} - M \\
m' & = M \left( \frac{B}{B'} - 1 \right) 
\end{align*}
\]  \hspace{1cm} (5)

RESULTS

Figure 2 shows the results of the albumin-coated charcoal assay of a B₁₂-deficient serum to which known quantities of crystalline B₁₂ have been added. The added B₁₂ ranged from zero to 1500 pg. per ml. of serum.

Figure 3 shows the albumin-coated charcoal assay of the B₁₂ content of different quantities of the same serum from a patient with chronic granulocytic leukemia. This serum contained 7190 pg. of B₁₂ per ml. The volume of serum assayed varied from 0.1 to 1.0 ml. The range of recovery was 98.3 to 103.3 per cent with a mean of 101.1 per cent. In addition this experiment demonstrates that a pathologic serum with a high B₁₂ level does not affect the accuracy of the assay.

Figure 4 compares the B₁₂ levels of 77 sera assayed by the Euglena gracilis® and albumin-coated charcoal methods. Each serum was tested in triplicate by the Euglena procedure and in duplicate by the charcoal method. The
Fig. 3.—Assay of varying quantities of a pathologic serum with high B12 concentration (7190 pg. per ml.) from a patient with chronic granulocytic leukemia. The assay is accurate for the abnormally high B12 level found in such a pathologic serum.

B12 levels ranged from less than 10 to more than 8000 pg. per ml. The B12 levels obtained for each serum by the two assays were sufficiently similar to permit the same diagnostic interpretation (see table 2). Values below 100 pg. per ml. were consistently lower in the charcoal assay. This may be due to the decreased sensitivity of the Euglena method in this range. Duplicate samples may vary by as much as 200 pg. in the Euglena assay, but vary less than 30 pg. in the charcoal assay.

Table 2 presents the interpretation of serum vitamin B12 levels assayed by either Euglena or albumin-coated charcoal in our laboratory. Since most values fall between zero and 1500 pg. per ml. any assay procedure should separate this range of vitamin B12 levels into easily recognized diagnostic groups. The quantity of Co57B12 used in the radiodilution method determines its sensitivity within a particular range. To meet these requirements 500 pg. Co57B12 is used in the method described for assay of 0.5 ml. of serum. This quantity of Co57B12 exceeds the B12 binding capacity of the NFIF by approximately 20 per cent, to ensure complete saturation of the B12 binding sites on the intrinsic factor concentrate. For maximum accuracy in measuring serum B12 levels between zero and 200 pg. per ml., the quantity of serum can be increased, or the amount of high specific activity Co57B12 decreased to 100 pg. with a proportional decrease of NFIF. Similarly, when assaying B12 levels greater than 1500 pg. per ml., the quantity of serum used can be decreased or the quantity of Co57B12 increased (the latter with a proportional increase.
Fig. 4.—B₁₂ levels of 77 sera as assayed by *Euglena gracilis* and albumin-coated charcoal are compared. Correlation is sufficiently close to permit similar diagnostic interpretation of each serum B₁₂ level.

The increased accuracy provided by such changes is unnecessary for diagnostic interpretation of serum B₁₂ levels.

**Discussion**

Herbert, Castro and Wasserman demonstrated that B₁₂ and IF were stochiometrically related, and suggested applying this relationship to a radioisotope dilution assay for B₁₂ in biologic fluids. Rothenberg and Barakat and Ekins used the principle of radioisotope dilution to assay serum B₁₂ levels. Rothenberg used IF concentrate to bind B₁₂, followed by protein precipitation to separate free from bound B₁₂. Barakat and Ekins used serum to bind B₁₂, followed by dialysis to separate free from bound B₁₂. Subsequent-

<table>
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<tr>
<th>pg. B₁₂ per ml. serum</th>
<th>Interpretation</th>
<th>Number of Samples Examined</th>
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<tr>
<td>&lt;100</td>
<td>Low</td>
<td>19</td>
</tr>
<tr>
<td>100–150</td>
<td>Suggestive of deficiency</td>
<td>3</td>
</tr>
<tr>
<td>150–200</td>
<td>Indeterminate</td>
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<td>900–1000</td>
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<td>0</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>High</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>77</strong></td>
</tr>
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</table>
ly, Grossowicz, Sulitzeanu and Merzbach\textsuperscript{12} reported an assay using serum to bind B\textsubscript{12}, and unaware of the coating effect of serum proteins, used charcoal to separate free from bound B\textsubscript{12}. Various procedures\textsuperscript{11,12} were used to prevent B\textsubscript{12} loss due to protein precipitation.

These methods showed that radioisotope dilution could be applied to assay of serum B\textsubscript{12} level. The present procedure avoids the difficulties of previous methods\textsuperscript{9,12} by the use of: (1) strong hydrochloric acid (0.25 N) and heat to free the bound B\textsubscript{12} in serum without protein coagulation; (2) intrinsic factor concentrate to bind the released B\textsubscript{12}, since it binds B\textsubscript{12} at low pH whereas serum cannot\textsuperscript{9,10}; (3) coated charcoal to provide effective and rapid separation of free from bound B\textsubscript{12}; (4) a control obviating the need for a calibration curve.

Bioassay procedures cannot be used easily with bacterially contaminated or turbid sera, and such procedures also give false low results if the serum contains a high level of many antibiotic and other noxious drugs. Charcoal assay does not have these disadvantages. The presence of autoantibodies to intrinsic factor in the test serum does not interfere with charcoal assay, because these antibodies are destroyed when the serum is heated in the boiling water-bath.

From equation (5) given earlier, it is clear that B’ can be solved for various anticipated values of B and m’ if all the values are considered as gravimetric units and expressed as percentages of M:

\[
\frac{B}{B'} - 1 = \frac{m'}{M}
\]

\[
B' = B \left( \frac{M}{m' + M} \right)
\]

Since B’ represents the percentage of Co\textsuperscript{57}B\textsubscript{12} that is bound to NFIF, its difference from 100 represents the percentage that is free or unbound. From these bound (B) and free (F) values, B/F ratios can be derived for any anticipated values of the NFIF control (B) and cold B\textsubscript{12} (m’). Table 3 shows B/F ratios calculated on this basis for NFIF control values of 40, 50, 60, 80, 90 and 100 per cent of added Co\textsuperscript{57}B\textsubscript{12} and for values of cold B\textsubscript{12} ranging from 0 to 300 per cent of Co\textsuperscript{57}B\textsubscript{12}. When B/F ratios are plotted against added cold B\textsubscript{12} values on arithmetic graph paper, typical radiodilution curves as described by Yalow and Berson\textsuperscript{13} for radioimmunoassay of insulin are obtained. Figure 5 shows the curves plotted from data in table 3. It can be seen that as the initial B/F ratio diminishes the curve flattens. The shape of the curve is determined by the initial B/F ratio. Experimental data obtained for various selected Co\textsuperscript{57}B\textsubscript{12} binding capacities of the NFIF control were essentially identical to these theoretical curves and can be superimposed on them. Figure 6 shows such a curve plotted from data used for figure 2. Comparison of figure 2 with figure 6 shows that values for the agents added may be plotted either as recovered agent (fig. 2) or as the ratio of bound agent to free agent (fig. 6). This suggested that the plotting of a series of known quantities of added agent, as had been done by Yalow and Berson\textsuperscript{13} in their radioisotope
Table 3.—Bound/Free Ratios Calculated for Different Percentages of Co²⁷B₁₂ Binding Capacity of the NFIF Control and Added Cold B₁₂

<table>
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<tr>
<th>Added Cold B₁₂ as Per Cent of Co²⁷B₁₂</th>
<th>Per Cent Bound</th>
<th>Per Cent Free</th>
<th>B/F</th>
<th>Per Cent Bound</th>
<th>Per Cent Free</th>
<th>B/F</th>
<th>Per Cent Bound</th>
<th>Per Cent Free</th>
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<tr>
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<td>16.0</td>
<td>84.0</td>
<td>0.19</td>
</tr>
<tr>
<td>200</td>
<td>20.0</td>
<td>80.0</td>
<td>0.25</td>
<td>16.7</td>
<td>83.3</td>
<td>0.20</td>
<td>13.3</td>
<td>86.7</td>
<td>0.15</td>
</tr>
<tr>
<td>300</td>
<td>15.0</td>
<td>85.0</td>
<td>0.18</td>
<td>12.5</td>
<td>87.5</td>
<td>0.14</td>
<td>10.0</td>
<td>90.0</td>
<td>0.11</td>
</tr>
</tbody>
</table>

dilution immunoassay for insulin, may not be necessary and, when the charcoal technic was applied to insulin assay, this proved to be the case. If instead of plotting B/F, the ratio B/B' is plotted against added cold B₁₂, a straight line is obtained (fig. 7). The slope of this line is constant; any deviation from this constant means that either the “hot” or the “cold” B₁₂ was not accurately calibrated. This calibration correction may be read directly from such a graph by extrapolation of the graph to intersect at the abscissa.

The procedure here reported may be adapted for assay of a wide range of vitamins, minerals, hormones and other materials. The only requirements to make such assay feasible are: (1) the agent must be adsorbable by charcoal when in the free form (in this connection, it should be noted that charcoal will adsorb small quantities of almost anything); (2) a binder for the agent must exist whose molecular weight and configuration is sufficiently different from that of the agent so that only the free agent and not the agent attached to its binder will be adsorbed by appropriately coated charcoal; (3) the agent to be assayed must be available in a radioactive or otherwise labeled form to serve as a marker. Markers which have been successfully used in “competitive inhibition” assays in the past have been color and radioactivity. The concepts applied to development of the serum B₁₂ assay here reported have already been extended to charcoal assay of serum iron and serum insulin. Thus it has already been shown that this assay procedure can be applied to a serum vitamin, a serum mineral and a serum hormone, and the implication is clear that the same procedure would be applicable for assay of other vitamins, minerals and hormones.
MEASUREMENT OF SERUM VITAMIN B\textsubscript{12} LEVEL

Fig. 5.—Theoretical radiodilution curves plotted from data in table 3. The shape of the curve is determined by the initial B/F.

It should be noted that the simple formula derived in this paper for calculating the serum B\textsubscript{12} level is identical to the original radioisotope dilution assay formula of Rittenberg and Foster,\textsuperscript{17} and the straight line relationship of figure 7 has been previously clearly delineated by Hales and Randle.\textsuperscript{18} A preliminary report of this work has been presented.\textsuperscript{19}

**Summary**

A method for measurement of serum B\textsubscript{12} level using radioisotope dilution and charcoal coated with albumin or hemoglobin is presented. Its advantages over previously described radioisotopic methods and the classical bioassay procedure are greater simplicity, rapidity and reproducibility. The sensitivity range appears to extend from the lowest to the highest B\textsubscript{12} values of normal
and pathologic sera. The materials for the assay are readily available and the procedure can be completed within an hour. The assay can be applied to other biologic fluids and materials and also readily adopted for routine use by any clinical laboratory equipped to use radioisotopes.

Summario in Interlingua

Es presentate un metodo pro le mesurement del concentration seral de Vitamina B₁₂, le qual utilisa un radioisotopo in dilution e carbon de ligno revestite de albumina o hemoglobina. Le avantages del metodo in comparation con previemente describite methodos radioisotopic e le bioessayage classic es que illo es plus simple, plus rapide, e plus uniformemente reproducibile. Le region de sensibilitate del metodo pare includer tanto le plus base como etiam le plus alte valores de B₁₂ incontrate in seros normal e in seros pathologic. Le requirite materiales es prestemente obtenibile, e le integre processo pote esser completate intra un hora. Le metodo es applicabile a liquidos
Measurement of Serum Vitamin B₁₂ Level

Fig. 7.—B/B' plotted against added cold B₁₂. B = Co¹⁷B₁₂ bound by NFIF control; B' = Co³⁷B₁₂ bound by NFIF after radiodilution by added cold B₁₂. A straight line (a) with a constant slope is obtained if the Co³⁷B₁₂ and cold B₁₂ are accurately standardized. If only 50 per cent of the expected weight of the Co³⁷B₁₂ is present, the degree of radiodilution is greater and a steeper slope is obtained as shown by line (b). Line (c) shows the reverse effect when 150 per cent of the expected weight of Co³⁷B₁₂ is present. The correct weight of Co³⁷B₁₂ present can be obtained by extrapolation of the graph to intersect the abscissa.

E materiales biologic altere que sero e se presta ben al uso routinari in laboratorio clinic equipate a travaliar con radioisotopos.

ACKNOWLEDGMENT

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REFERENCES


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Measurement of Serum Vitamin B$_{12}$ Level Using Radioisotope Dilution and Coated Charcoal

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