Brief Report

Coated Charcoal Assay of Erythrocyte Vitamin B\textsubscript{12} Levels

By Alan Kelly and Victor Herbert

When erythrocyte vitamin B\textsubscript{12} levels are determined by the coated charcoal method of Lau et al.,\textsuperscript{1} the proteins precipitate during heating, creating a gelatinous mass which interferes with carrying out the rest of the protocol. This report presents a modification of the original protocol which eliminates this problem.

Erythrocyte vitamin B\textsubscript{12} levels are of special clinical value when the serum vitamin B\textsubscript{12} level is artificially high due to chronic myelogenous leukemia, liver disease, or a recent injection of vitamin B\textsubscript{12}. Since vitamin B\textsubscript{12} enters mainly reticulocytes\textsuperscript{2,4} and not mature erythrocytes, the erythrocyte vitamin B\textsubscript{12} level may be a more legitimate measure of tissue B\textsubscript{12} stores in the presence of artificial elevation of the serum level.

\textbf{B\textsubscript{12} Extraction}

The methodology of Spra\textsuperscript{5} for extracting vitamin B\textsubscript{12} from serum is applied to extracting the vitamin from erythrocytes, as previously reported.\textsuperscript{2,6} To 2 ml. of packed unwashed erythrocytes, add 2 ml. of 0.4M acetate buffer (pH 4.9 $\pm$ 0.4), 0.4 ml. of 0.1 per cent (w/v) NaCN solution, and 15.6 ml. distilled water. Autoclave at 118 C. for 15 minutes, cool, centrifuge, remove clear pale pink supernatant, which is the extract.

\textbf{Coated Charcoal Assay (Table 1)}

All glassware should be scrupulously clean and free from traces of vitamin B\textsubscript{12}. The assay is carried out in duplicate in 10 ml. test tubes. All reagents are standardized as described by Lau et al.\textsuperscript{1}

Two (2.0) ml. of the unknown extract was added to a test-tube. The Intrinsic Factor, Supernatant Control, and Standard tubes contained 2.0 ml. of 0.9 per cent saline. These were run parallel to the unknowns. To all tubes 0.1 ml. of a solution containing 1.0 ng. Co\textsuperscript{57}B\textsubscript{12} per ml. was added. This was mixed thoroughly, and then 0.1 ml. of National Formulary Intrinsic Factor Concentrate (NFIF)\textsuperscript{*} was added to the unknown and intrinsic factor

\textsuperscript{*}Purchased as "Intrinsic Factor, 5 \times N.F. without B\textsubscript{12}" from Nutritional Biochemicals Corp., Cleveland, Ohio, at $13.50 for 50 mg. This powder is diluted in 0.9 per cent saline to 5 \mu g. NFIF per ml. saline. Any other hog intrinsic factor concentrate may be used instead of NFIF, provided the quantity used is that amount which will bind between 60 and 80 per cent of 100 pg. of Co\textsuperscript{57}B\textsubscript{12}. The quantity is determined by running a curve, as in Figure 1 of reference 7.
Table 1.—Erythrocyte Vitamin B₁₂ Assay Protocol Sequence of Addition and ml. of Reagents

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unknown</th>
<th>Saline 0.9% Co₂H₂</th>
<th>1000 µg./ml. N.F.I.F. *</th>
<th>Hemoglobin-Coated Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>2.0</td>
<td>—</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>NFIF*</td>
<td>—</td>
<td>2.0</td>
<td>0.1</td>
<td>Mix well</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td>Centrifuge at 3000 rpm for 15 minutes.</td>
</tr>
<tr>
<td>Supernatant control</td>
<td>—</td>
<td>2.0</td>
<td>0.1</td>
<td>Mix well</td>
</tr>
<tr>
<td>Standard</td>
<td>—</td>
<td>2.0</td>
<td>2.1</td>
<td>—</td>
</tr>
</tbody>
</table>

* NFIF = National Formulary Intrinsic Factor.

control tubes, which are mixed thoroughly and allowed to stand for 30 minutes. Two ml. of hemoglobin-coated charcoal, prepared as previously described, were added to all tubes except the Standard tubes. The volume of the supernatant and standard tubes was made up to 4.2 ml. with 0.9 per cent saline. All tubes were thoroughly mixed and centrifuged at 3000 rpm for 15 minutes. The supernatants were decanted into counting tubes and their radioactivity determined using a well type scintillation detector.

**Calculation**

The counts per minute (cpm) of the supernatant control were subtracted from those of the unknown and intrinsic factor control to obtain the net counts. The vitamin B₁₂ level of the erythrocytes was determined by the following formula:

\[
p_{B_{12}} \text{ pg. per ml. packed erythrocytes} = 5 \times \text{pg. Co}^{57} B_{12} \left( \frac{B}{B'} - 1 \right)
\]

Where \(B\) = the net cpm of Intrinsic Factor control tube

\(B'\) = the net cpm of unknown tube

The multiplication by 5 compensates for the fact that the assay is performed on 2 ml. of extract (which is derived from 0.2 ml. of erythrocytes).

**Microbiologic Assay**

The extracts were assayed microbiologically using *Euglena gracilis* as the test organism.

**Results**

Fifty erythrocyte extracts were assayed by both assay technics for vitamin B₁₂ content. The results with the coated charcoal assay ranged from 72 pg./ml. packed erythrocytes to 520 pg./ml. packed erythrocytes, with a mean of 209 pg./ml. packed erythrocytes. With the *Euglena* assay, results were from 72 pg./ml. packed erythrocytes to 512 pg./ml. packed erythrocytes, with a mean of 205 pg./ml. packed erythrocytes. Figure 1 gives a comparison of the results obtained by the two methods.

The recovery of added vitamin B₁₂ at the 500 pg. level was from 68 per cent to 115 per cent, with a mean of 91 per cent for the coated charcoal assay, compared with a range of 61 per cent to 114 per cent, with a mean of 87 per cent for the *Euglena* assay.

One extract was assayed on 8 different occasions. The results ranged from 76
COATED CHARCOAL ASSAY OF ERYTHROCYTE VITAMIN B₁₂ LEVELS

Fig. 1.—Erythrocyte vitamin B₁₂ levels (pg./ml.) in 50 normal subjects as determined by microbiologic assay vs. hemoglobin-coated charcoal assay.

to 105 pg. B₁₂/ml. packed erythrocytes, with a mean of 91 pg. B₁₂/ml. packed erythrocytes.

Table 2 shows that the level of B₁₂ in erythrocytes of normal subjects may vary from about a third to as much as about 90 per cent of the serum level.

DISCUSSION

In 1950, Couch et al.⁹ reported B₁₂ content of whole blood from 10 humans as ranging from 600 to 1400 pg./ml., with an average of 800 pg./ml. by L. Leichmannii assay. Rosenthal and Brown,¹⁰ using the same assay, reported

<table>
<thead>
<tr>
<th>Plasma Vitamin B₁₂ (pg./ml.)</th>
<th>Erythrocytes</th>
<th>Erythrocyte B₁₂ as Per Cent of Serum Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Charcoal</td>
<td>Charcoal</td>
</tr>
<tr>
<td>392</td>
<td>212</td>
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<tr>
<td>307</td>
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<td>539</td>
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<td>200</td>
</tr>
<tr>
<td>501</td>
<td>202</td>
<td>164</td>
</tr>
</tbody>
</table>

Avg. 392  211  194  58  56
human whole blood B12 levels as averaging 260 pg./ml. (average of six samples), and plasma values as averaging 160 pg./ml. (five samples). Using *L. Leichmannii*, Kato reported the average B12 levels of 20 normal males to be 195 pg./ml. in the red cells and 362 pg./ml. in the plasma, with red cell values varying from 135 to 230 pg./ml. He also reported that B12 in human erythrocytes is released from protein binding more readily in pH 4.6 acetate buffer than pH 6.8 phosphate buffer, and mentioned unpublished experiments in which he had found the vitamin content of rabbit erythrocytes markedly increased in active regeneration following anemia, in proportion to the intensity of regeneration, with the increase being mainly due to abundance of the vitamin in the soluble fraction of newly generated erythrocytes; the increase declined rapidly with recovery from anemia. He further reported that the vitamin B12 content of the whole blood cell mixture was not changed significantly by removal of the overlyinguffy coat, including leukocytes and platelets, suggesting that almost all of the vitamin activity of the cellular fraction of normal human blood came from the erythrocytes (or at least that leukocytes and platelets do not contribute an inordinately large percentage of the total B12 level of the packed cell fraction of normal blood).

Using *L. Leichmannii* assay, Herbert reported, “As vitamin B12 deficiency develops, the serum vitamin B12 level falls first and is slowly followed by the red cell vitamin B12 level. Thus, in five patients whose serum vitamin B12 levels had fallen to an average of 34 pg./ml., the vitamin B12 content of the unwashed red cells still averaged 140 pg./ml.”

Sobotka, et al. reported red cell B12 levels of three normal subjects to average 165 pg./ml. They found the average red cell B12 level of five patients with B12 deficiencies to be 92 pg./ml.

Using *L. Leichmannii*, Biggs et al. found the B12 level of the red cells of 50 normal subjects to range from 110 to 500 pg./ml., with a mean of 213 pg./ml.; the red cell B12 of twelve patients with untreated pernicious anemia ranged from 25 to 110 pg./ml., with a mean of 63 pg./ml.; 8 patients with “latent pernicious anemia” had red cell B12 values ranging from 62 to 210 pg./ml., with a mean of 119 pg./ml.

Thus, the coated charcoal assay for red cell B12 levels gives results consistent with those of prior studies using various microbiologic assays.

We have recently seen two patients with serum B12 levels of 77 and 136 pg./ml.*, but no anemia, overt megaloblastosis, or stigmata of B12 deficiency other than an increased lobe average of the nuclei of the neutrophils in the former patient. Both patients had normal erythrocyte B12 levels (144 and 188 pg./ml.), adding to the evidence that erythrocyte B12 levels more accurately reflect tissue stores of B12 than do serum B12 levels, in man.

This same methodology may be used to assay liver B12. In a preliminary study, we found liver B12 to be 0.26 µg./Gm. by coated charcoal assay as compared to 0.29 µg./Gm. by *E. gracilis* assay in a pernicious anemia patient who

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*In our laboratory, serum B12 levels of 100–200 pg./ml. are equivocal; 200 is our lower limit of normal and 100 is our upper limit of unequivocal B12 deficiency.*
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had for 10 years been treated with oral B₁₂. This patient with low liver B₁₂ stores, despite therapy, will be separately reported by E. Brody.

SUMMARY

A rapid, reproducible coated charcoal assay for vitamin B₁₂ in erythrocytes is described. Results are almost identical to those of microbiologic assay.

SUMMARY IN INTERLINGUA

Es describite un rapid e reproducibile methodo de essayage a revestim ento carbonic pro vitamina B₁₂ in erythrocytos. Le resultatos es quasi identic con illos obtenibile per essayage microbiologic.

ACKNOWLEDGMENTS

We are indebted to Misses Leona Bandel, Le Teng Go, and Melody Lee for aid in this study.

REFERENCES

Brief Report: Coated Charcoal Assay of Erythrocyte Vitamin B$_{12}$ Levels

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