Measurement of the Amounts of the Individual Vitamin B\textsubscript{12} Binding Proteins in Plasma

I. Studies of Normal Plasma

By Charles A. Hall and Alexander E. Finkler

The vitamin B\textsubscript{12} in blood is bound to protein.\textsuperscript{1} With the first identification of a specific B\textsubscript{12} binding protein,\textsuperscript{2} it became obvious that more than one serum protein could bind B\textsubscript{12} and that the participating proteins were not the same for naturally circulating B\textsubscript{12} and B\textsubscript{12} added to serum in vivo or in vitro.\textsuperscript{3,4} The transport functions of the individual serum binders remain unclear but at least two of them, transcobalamin I (TC I) and transcobalamin II (TC II), appear to function in the normal metabolism of vitamin B\textsubscript{12}.\textsuperscript{5,6}

Cells are not indifferent to the form in which vitamin B\textsubscript{12} is presented to them. The need for intrinsic factor for the uptake of B\textsubscript{12} by the cells of the intestinal mucosa is well-known, and the state of B\textsubscript{12} in plasma also influences uptake by cells. Body tissues remove vitamin B\textsubscript{12} from plasma much more rapidly if it is bound to TC II than to TC I.\textsuperscript{6} The carrier of B\textsubscript{12} is important in another, somewhat artificial cell system, the HeLa cell in tissue culture. Very little B\textsubscript{12} is taken up by the cells if it is bound to TC I or if it is in a free state, while uptake is much enhanced by binding with TC II.\textsuperscript{7}

Starting with this clear demonstration of the vital importance of the plasma binders of B\textsubscript{12}, we planned the present study for the detection of the different binding substances in plasma, for the measurement of their capacity to bind B\textsubscript{12} and for the detection of abnormalities in disease.

The first step, which is presented in Part I, was to devise the proper technic. Earlier methods measured only the total capacity of serum to bind B\textsubscript{12}. Several basic methods have been established, each using a different principle to measure the B\textsubscript{12} which was bound.\textsuperscript{5,8-10} The failure of these methods to differentiate binding by the several substances is probably the basis for the differences in results obtained by them and also made them unsuitable for the present study. However, a system devised previously\textsuperscript{5,6} in this laboratory for the separation of TC I and TC II after they had been labeled with B\textsubscript{12} in vivo was found to be applicable to the present in vitro study. The basic principles of the technic used here were the addition of Co\textsuperscript{57} B\textsubscript{12} to plasma, the removal of unbound B\textsubscript{12} by dialysis, the separation of the binding proteins by column chromatography and the measurement of the B\textsubscript{12} bound to each.

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METHODS

The same techniques were used in both Parts I and II of this study. Co\(^{57}\)B\(_{12}\) cyanocobalamin was used exclusively and almost all of it, which had an initial specific activity of 5-10 \(\mu\)c./\(\mu\)g., was obtained from a single source. Co\(^{57}\)B\(_{12}\) with an initial specific activity of over 100 \(\mu\)c./\(\mu\)g. was used in a few cases. The specific activity was reduced with USP Reference Standard nonradioactive cyanocobalamin when large amounts of B\(_{12}\) were added to plasma. Each lot of Co\(^{57}\)B\(_{12}\) was checked for B\(_{12}\) content by bioassay with Englella gracilis, Z strain.

The B\(_{12}\) was added to 3-20 ml heparinized plasma which sometimes was fresh but usually had been stored at about 20 \(^\circ\)C. The presence of heparin gave no concern initially since it had been shown that it would not bind with cyanocobalamin.\(^{11}\) Moreover, we have always obtained similar binding patterns in the in vitro studies as in the previous in vivo studies where the heparin was not added until the B\(_{12}\) was firmly bound to plasma proteins. Recently, it was stated that heparin will bind vitamin B\(_{12}\) presumably cyanocobalamin.\(^{12}\)

To evaluate potential binding by heparin, identical amounts of cyanocobalamin were added to first heparinized plasma and then serum from the same subject. The patterns of binding to the plasma proteins were the same and it was concluded that no artifacts were introduced by the heparin.

The amounts of B\(_{12}\) added ranged from 1.0 pg. per ml. of plasma to 20 ng. per ml. The picogram (pg.) is equal to the better-known unit, micromicrogram (\(\mu\)ug.) and 1 nanogram (ng.) is equal to 1 millimicrogram (\(\mu\)ug.). One ng. = 100 pg. The B\(_{12}\) was added in a volume of 10-20 per cent of that of the plasma sample and was incubated with the plasma for 20 minutes at 37 \(^\circ\)C. The mixture was dialysed in the cold for 48 hours against 3 changes of the starting buffer used for the column chromatography. The plasma proteins were separated by DEAE anion-exchange column chromatography by a technic previously described in detail.\(^{8}\) The chromatogram of the Co\(^{57}\)B\(_{12}\) and the protein contained in each fraction of eluate from the column was plotted. The limits of the two binding proteins, TC I and TC II, were located on the plot according to previous standards of localization.\(^{6}\) and the amount of added B\(_{12}\) bound to each protein was calculated from the sum of Co\(^{57}\)B\(_{12}\) found in all fractions of the eluate falling within the limits of each protein. The control plasma was taken from the two authors and two patients with mild hemochromatosis.

RESULTS

The data are presented in a uniform manner. The added B\(_{12}\) bound to each protein is expressed as pg. of added B\(_{12}\) per ml. of plasma. This is plotted as pg. per fraction of the eluate per ml. of plasma in the figures and as total pg. per binding protein per ml. of plasma in the tables. The peak of TC II falls at about fraction #120 in all of the chromatograms and that of TC I at about #210. The bulk of the plasma protein, which consists mainly of albumin, shows a sharply rising peak at fraction #170-180 with a more gradual tailing off. The data in the tables are only semiquantitative because of the difficulty in accurately defining the limits of each protein in the chromatograms. Quantitation was much poorer with the larger amounts of added B\(_{12}\) as compared with 1-300 pg./ml. because the greater number of proteins participating in the binding could not be cleanly separated.

Studies with “Subcapacity” Amounts of Cyanocobalamin

Much of the work was done with 300 pg./ml. of added B\(_{12}\), an amount which corresponded to the increase in plasma B\(_{12}\) induced by the in vivo studies done

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[1] Purchased from Radiochemical Centre, Amersham, England through the courtesy of Merck Chemical Division, Merck and Co., Inc.
Fig. 1.—Comparison of two levels of cyanocobalamin added to samples of plasma from control subject A. F. The spreading of the protein in Figure 1a is probably the result of using 20 ml. of plasma made necessary by the small amount of B12.

previously in this laboratory. At this level of added B12, the greater part of the added B12 was eluted with a prealbumin protein which we call TC II. A typical chromatogram is shown in Figures 1b and 2a. The first 89 fractions containing the gamma globulins and some of the beta globulins are not illustrated since we did not find B12 in this region. A small peak was seen in the alpha globulin region which we call TC I. We found the endogenous B12 to be in this region in the previous in vivo studies. Although some added B12 was always present in the region of the main protein peak, it was unusual to find a distinct peak as in one subject, Figure 1b. The relative binding among these substances for the four plasmas studied is shown in Table 1. Since the addition of 300/pg./ml. of plasma may double the plasma B12 level, we studied the binding pattern of an amount which was no more than 0.2 per cent of the plasma level of B12 and probably represented a truly tracer amount. One pg./ml. was added to the plasma of one control (Fig. 1a). The binding pattern was essentially the same as that of the larger amount (Fig. 1b) although there was less spreading of the B12 at the 1.0 pg. level.
Studies with “Overcapacity” Amounts of Cyanocobalamin

In order to test the capacity of the two B₁₂ binding proteins, amounts of 1.5, 5.0 and 10.0 ng./ml. of B₁₂ were added, followed by dialysis and then separation of the proteins. A typical chromatogram after 1.5 ng./ml. (Fig. 2b) shows that the amounts bound to TC I and TC II retained about the same relationship as when smaller amounts were added. However, with the large amounts there was a B₁₂ peak in the region of the bulk of the plasma proteins. The semiquantitative data from the four control subjects is shown in Table 2. TC I and TC II participated in the binding at all levels of B₁₂, but with large amounts other proteins became involved. The studies with subjects C. H. and R. H. (Table 2) suggest a plateau affect for TC I and II but not for binding in the main protein region.

DISCUSSION

The major binding protein of 1.0 or 300 pg. amounts of added B₁₂ was transcobalamin II or the same major binder of B₁₂ recently injected or ab-
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Table 1.—Co\(^{12}\) Cyanocobalamin Bound to Plasma Fractions after 300 pg. ml. Plasma (Control Subjects)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Per Cent Bound</th>
<th>TC I</th>
<th>Main Protein Peak</th>
<th>TC II</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. F.</td>
<td>90</td>
<td>27</td>
<td>13</td>
<td>79</td>
</tr>
<tr>
<td>C. H.</td>
<td>83</td>
<td>22</td>
<td>35</td>
<td>110</td>
</tr>
<tr>
<td>R. H.</td>
<td>95</td>
<td>28</td>
<td>0</td>
<td>140</td>
</tr>
<tr>
<td>A. N.</td>
<td>94</td>
<td>26</td>
<td>0</td>
<td>150</td>
</tr>
</tbody>
</table>

Table 2.—Co\(^{12}\) Cyanocobalamin Bound to Plasma Fractions with Increasing Amounts Added (Control Subjects)

<table>
<thead>
<tr>
<th>Amount Added</th>
<th>Per Cent Bound</th>
<th>TC I</th>
<th>Main Protein Peak</th>
<th>TC II</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. F.</td>
<td>1.0 pg./ml.</td>
<td>87</td>
<td>0.02</td>
<td>0.51</td>
</tr>
<tr>
<td>300.0 pg./ml.</td>
<td>90</td>
<td>27</td>
<td>14</td>
<td>79</td>
</tr>
<tr>
<td>5,000.0 pg./ml.</td>
<td>22</td>
<td>170</td>
<td>610</td>
<td>220</td>
</tr>
<tr>
<td>C. H.</td>
<td>0.3 ng./ml.</td>
<td>83</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>1.5 ng./ml.</td>
<td>61</td>
<td>92</td>
<td>200</td>
<td>340</td>
</tr>
<tr>
<td>5.0 ng./ml.</td>
<td>40</td>
<td>54</td>
<td>300</td>
<td>330</td>
</tr>
<tr>
<td>R. H.</td>
<td>0.3 ng./ml.</td>
<td>95</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>1.0 ng./ml.</td>
<td>55</td>
<td>350</td>
<td>1,000</td>
<td>320</td>
</tr>
<tr>
<td>10.0 ng./ml.</td>
<td>52</td>
<td>220</td>
<td>2,700</td>
<td>560</td>
</tr>
<tr>
<td>A. N.</td>
<td>0.3 ng./ml.</td>
<td>94</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>10.0 ng./ml.</td>
<td>57</td>
<td>680</td>
<td>2,300</td>
<td>1,100</td>
</tr>
</tbody>
</table>

sorbed.\(^6\) This probably was the same substance as the beta-globulin binder observed by others\(^2,3,13\) and the plasma "R" of Simons.\(^14\) The small amount of binding in the alpha-globulin region of the chromatogram corresponded to the transcobalamin I of our in vivo studies.\(^6\) It appeared to be the same as the alpha-globulin binding of native vitamin B\(_{12}\) observed by others\(^2,15,17\) and the B\(_{12}\) "BP" of Miller and Sullivan.\(^4\) We were unable to determine whether the binding protein which binds native B\(_{12}\) was identical to that which took up a small amount of added B\(_{12}\); that is, we could not be sure that TC I was a single substance. The chromatograms after oral and injected B\(_{12}\)\(^6\) suggested that there was only one B\(_{12}\) binder in the TC I region. However, further processing of the fractions in this region has given a hint that in fact there may be two binders. The technical problems here are considerable and much more work is needed for a final answer. The protein or proteins which also bind B\(_{12}\) when it is added in very large amounts have not been identified. Since the peak of added B\(_{12}\) bound to these proteins corresponded to the largest protein peak of plasma, we have simply related it to the main protein peak.

Our data clearly showed a change in the binding pattern with increasing amounts of added B\(_{12}\). At 0.3 ng./ml and below, TC II was the principal binder with TC I playing a minor role, and we consider these to be "primary
binders.” Proteins of the main protein region dominated at 5 or 10 ng./ml. One control plasma (A.F.) showed some “nonspecific” or “secondary” binding at the 0.3 ng./ml. level, while another (A. N.) bound very little to these proteins at 1.0 ng./ml. The data from subject C. H. suggests that the change in binding pattern became evident between 0.3 and 1.5 ng./ml.

The results of this study fit nicely with much of the previous work of others. The concept of primary and secondary plasma B12 binders has been presented previously\textsuperscript{13,19} without identification of the substance. However, there is evidence that the primary binders are in the seromucoid fraction of plasma protein while the secondary binders are not.\textsuperscript{13,19,20}

CONCLUSIONS

The findings of the present study, when combined with those of previous studies, support the following concept of binding of vitamin B12 by serum or plasma in vitro. There are primary binders of B12 which preferentially take B12 when small amounts are added and combine firmly with it. They include two already identified substances, TC I and TC II, which are of the seromucoid fraction of plasma. At concentrations of added B12 of the order of 1.0 ng./ml. of plasma, these proteins become saturated and secondary binders become important. The latter are relatively unimportant at lower concentrations of B12; they are weak binders of B12; they cannot be saturated by concentrations used to date, and they are not seromucoids.

SUMMARIO IN INTERLINGUA

Le constatazioni del presente studio, quando combine con illos de previe studios, supporta le sequente conception del ligation de vitamina B12 per sero o plasma in vitro. Il existe ligatores primari de vitamina B12 que accepta B12 preferentialmente quando micre quantitates es addite e que se combina firmemente con illo. Iste ligatores include duo jam identificate substantias, TC I e TC II, que pertine al fraction seromucoide de plasma. A concentrationes del addite vitamina B12 in le ordine de 1,0 ng (nonagramma) per ml de plasma, iste proteinas deveni saturate, e ligatores secundari deveni importante. A plus basse concentrationes de vitamina B12, iste ultimes es relativemente in-importante. Illos es debile ligatores de vitamina B12. Illos non pote esser saturate per concentonones usate usque a iste tempore, e illos non es seromucoides.

REFERENCES

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