THE SERUM IN CHRONIC MYELOGENOUS LEUKEMIA (CML) HAS AN INCREASED CONCENTRATION OF VITAMIN B₁₂ (1, 2) AND AN INCREASED CAPACITY TO BIND ADDED VITAMIN B₁₂ EITHER IN VIVO (3–5) OR IN VITRO (2, 6, 7). THE VITAMIN B₁₂ OF NORMAL AND CML SERUM MOVES WITH THE α-GLOBULINS WHEN SERUM IS ELECTROPHORESED AT pH 8.6 (1, 8, 9). THE SEROMUCOID, THE FRACTION OF SERUM CONTAINING GLYCOPROTEINS WHICH COMPRISSES ABOUT 1.5 PER CENT OF THE SERUM PROTEINS (10), MOVES WITH THE α-GLOBULINS WHEN ELECTROPHORESED AT pH 8.6 (11). FURTHERMORE, IT HAS BEEN FOUND THAT THE SEROMUCOID FRACTION ISOLATED FROM CML SERUM IS CAPABLE OF BINDING AMOUNTS OF ADDED COBALT-Labeled VITAMIN B₁₂ (B₁₂*) SUFFICIENT TO ACCOUNT FOR THE INCREASED BINDING CAPACITY OF THE WHOLE SERUM (12). THE POSSIBILITY THAT THE BINDING PROTEIN OF BOTH NORMAL AND CML SERUM FOR NATIVE VITAMIN B₁₂ (B₁₂BP) IS IN THE SEROMUCOID FRACTION WAS INVESTIGATED.

METHODS

Venous blood was collected in the fasting state from normal subjects and from patients with CML in hematological and clinical relapse. About one to two hours after clotting, the serum was separated and either used immediately or stored at –20°C. The seromucoid fraction of serum was prepared by the method of Winzler, Devor, Mehl and Smyth (10) as follows: one volume of serum was added to one volume of 0.4 M sulfosalicylic acid, thoroughly mixed for five minutes, and then centrifuged. The supernatant containing the seromucoid was separated and neutralized with 1 N NaOH. The neutralized seromucoid and the original serum from which it had been prepared were dialyzed against large volumes of 0.15 M NaCl for 72 hours at 4°C. and its vitamin B₁₂ concentration then determined by Euglena gracilis assay (13). When used for electrophoresis, the seromucoid was dialyzed for 72 hours against large volumes of distilled water, lyophilized, and the dried protein then dissolved in the appropriate buffer. Seromucoid or serum containing bound (nondialyzable) B₁₂ was prepared by adding an amount of B₁₂ in excess of the binding capacity (4 µg. B₁₂ per ml. of normal serum or its equivalent seromucoid and 12 µg. per ml. of CML serum or its equivalent seromucoid). The seromucoid was then dialyzed against large volumes of distilled water for 72 hours at 4 to 6°C. and lyophilized, while the serum was dialyzed against 0.15 M NaCl.

Lyophilized Cohn plasma fractions (I through VI), prepared by Method VI (14), were dissolved in sufficient 0.15 M NaHCO₃ to bring them to their equivalent plasma volumes, dialyzed for 48 hours against 0.15 M NaCl, and the vitamin B₁₂ concentration determined as was the vitamin B₁₂ concentration of the original plasma.

Zone electrophoretic separation of serum or lyophilized seromucoid was performed using starch blocks as the supporting medium. The general technique described by Kunkel (15) was used except that the electrophoresis was carried out at pH 4.5 which permitted separation of the B₁₂BP from the bulk of serum proteins. The starch blocks were prepared from slurries of starch that had been previously washed once with distilled water and twice with an acetate-sodium chloride buffer pH 4.5, ionic strength 0.10. The buffer consisted of 0.04 M sodium acetate and 0.06 M NaCl with concentrated HCl added to bring the pH to 4.5. The following samples were electrophoresed: a) a mixture of 1.0 ml. of serum and 0.5 ml. of acetate buffer; b) serum containing bound (nondialyzable) B₁₂ in a volume of 1.5 to 2 ml.; c) lyophilized seromucoid dissolved in a solution of 1 ml. of 0.15 M NaCl and 0.5 ml. acetate buffer; and d) lyophilized seromucoid containing bound B₁₂ dissolved in c. Samples were applied to a 4 to 5 mm. transverse slit at the center of the block. In some experiments, serum containing bound B₁₂ was run in parallel on the same starch block as the native serum. Electrophoresis was carried out for 16 hours at 5 to 8°C. using a current flow of 36 ma. for a 31 cm. long, 10 cm. wide, 1.5 cm. thick starch block. At the termination of electrophoresis a maximum

1 Kindly supplied by Dr. N. S. Ritter, Merck and Co., Rahway, N. J.
2 Kindly prepared by the Plasma Protein Foundation, Boston, Mass.
TABLE I

The native vitamin B\textsubscript{12} concentration of the seromucoid of normal and chronic myelogenous leukemic (CML) sera

<table>
<thead>
<tr>
<th>Subject</th>
<th>Serum B\textsubscript{12} concentration</th>
<th>Seromucoid B\textsubscript{12} concentration</th>
<th>Seromucoid fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu\text{g./ml.})</td>
<td>(\mu\text{g./ml.})</td>
<td>%</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S.W.</td>
<td>525</td>
<td>435</td>
</tr>
<tr>
<td>2</td>
<td>L.S.</td>
<td>125</td>
<td>122</td>
</tr>
<tr>
<td>3</td>
<td>A.N.</td>
<td>732</td>
<td>451</td>
</tr>
<tr>
<td>4</td>
<td>M.B.</td>
<td>732</td>
<td>539</td>
</tr>
<tr>
<td>5</td>
<td>T.C.</td>
<td>681</td>
<td>544</td>
</tr>
<tr>
<td>6</td>
<td>S.J.</td>
<td>332</td>
<td>208</td>
</tr>
</tbody>
</table>

| Mean    | 521                     | 383                 | 77      |

CML

| 1       | H.W.                    | 6,096               | 5,976   | 98  |
| 2       | A.M.                    | 15,121              | 15,101  | 100 |
| 3       | C.M.                    | 9,220               | 9,010   | 98  |

Results

Vitamin B\textsubscript{12} concentration of the seromucoid

The seromucoid contained an average of 77 per cent, range 62 to 98 per cent, of the vitamin B\textsubscript{12} of whole normal serum (Table I). Despite a great increase in serum vitamin B\textsubscript{12} concentration, 98 to 100 per cent of the vitamin was recovered from the seromucoid in three patients with CML.

Vitamin B\textsubscript{12} concentration of Cohn plasma fractions

Vitamin B\textsubscript{12} was found in all six Cohn plasma fractions. Fraction V contained the highest percentage of the plasma vitamin (27 per cent) while Fraction VI had only 14 per cent.

Vitamin B\textsubscript{12} concentration of fractions of the seromucoid separated by electrophoresis

The distribution of native vitamin B\textsubscript{12}, protein and sialic acid in the seromucoid, obtained from normal serum following starch gel electrophoresis at pH 4.5, is shown in Figure 1. The protein and sialic acid were concentrated in segments anodal to the origin with peak levels found in the A-3 and A-4 segments. A similar distribution of protein and sialic acid was found when a crystalline preparation of the acidic \(\alpha_1\)-glycoprotein was electrophoresed at pH 4.5. Acidic \(\alpha_1\)-glycoprotein, isolated from Cohn Fraction VI (19), was dissolved in a solution composed of 1 ml. 0.15 M NaCl and 0.5 ml. acetate buffer, electrophoresed on a starch block (pH 4.5) and the protein

4 Kindly supplied by Dr. Karl Schmid, Massachusetts General Hospital, Boston, Mass.

pH change of 0.2 pH unit was found at either end of the gel. After drying at room temperature (0.5 to one hour) the gel was cut into transverse segments so that the origin was included in one segment (O segment). Other segments were designated as anodal (A) or cathodal (C) and identified by the number of segments from the O segment. Sections of the block at the cathodal and anodal ends were removed. The range of migration of the proteins was removed to serve as blanks. Each segment was suspended in 8 ml. of distilled water, the contents periodically mixed for 1.5 hours, and then centrifuged. The supernatant was removed and centrifuged again. The vitamin B\textsubscript{12} concentration of these eluates was then determined by E. gracilis assay. The per cent recovery of vitamin B\textsubscript{12} from the starch block in 19 electrophoretic analyses of serum or seromucoid was equal to an average of 93 per cent ± 14, range, 75 to 115 per cent.

The protein concentration of starch eluates was determined by Kunkel and Tiselius' modification (16) of the Folin-Ciocalteu procedure (17). Protein concentration was plotted in terms of optical density with necessary corrections made for the volume of eluate analyzed. Sialic acid was determined by the method of Werner and Odin (18) using 2 ml. of the eluates. Because of the negligible reading of the blank with Baker's iso-amyl alcohol, further purification of the iso-amyl alcohol was not necessary.

In preliminary experiments, it was found that albumin and the bulk of the globulins remained at the origin or migrated cathodally when serum was electrophoresed at pH 4.5. Acidic \(\alpha_1\)-glycoprotein, isolated from Cohn Fraction VI (19), was dissolved in a solution composed of 1 ml. 0.15 M NaCl and 0.5 ml. acetate buffer, electrophoresed on a starch block (pH 4.5) and the protein
phoresed at pH 4.5. The vitamin B₁₂ was also found anodally with peak concentrations at A-3 to A-5 segments. The B₁₂* bound to the normal seromucoid had a distribution similar to the native vitamin, with peak concentrations at A-2 to A-4 segments (Figure 2).

The distribution of protein, sialic acid and vitamin B₁₂ in the CML seromucoid was similar to that of the normal, with peak concentrations found at A-3 to A-4 segments (Figure 3). Furthermore, the distribution of added B₁₂* bound by CML seromucoid was similar to that of the native vitamin, with peak concentrations located at A-2 to A-4 segments (Figure 4).

**Vitamin B₁₂ concentration of fractions of serum separated by electrophoresis**

Electrophoresis of a normal serum at pH 4.5 (Figure 5) is representative of the findings in a group of eight such sera. In sharp contrast to the seromucoid, there was a negligible amount of protein in A-2 to A-6 segments with the peak pro-
tein concentration at the 0 segment (A-1 or C-1 in some sera). However, virtually all of the native vitamin B₁₂ was recovered anodally (83 to 92 per cent found in segments A-2 to A-6), with peak concentrations in A-3 to A-5 segments. The distribution of the native vitamin B₁₂ and of the nondialyzable B₁₂* bound to the same serum is shown in Figure 6 and is representative of the findings in a group of seven such sera. The native vitamin had the same anodal distribution as previously described. Only a small amount of B₁₂* was found anodally, segments A-2 to A-6 containing an average of 80 μg per ml. ± 17, range, 28 to 183 μg per ml. The proteins with the greatest binding ability for the added B₁₂* were found at the origin and C-1 to C-3 segments. The binding of B₁₂* by A-2 to A-6 segments was equal to an average of 6 per cent ± 4, range, 3 to 15 per cent of the total bound radiovitamin. The saturation of the B₁₂BP (located at A-2 to A-6 segments) as estimated from the ratio of the native bound vitamin to the total vitamin that could be

![Graph](image-url)

**Fig. 3. Native Vitamin B₁₂ Concentration of Fractions of Chronic Myelogenous Leukemia Seromucoid Separated by Electrophoresis on Starch Gel at pH 4.5.**

Vitamin B₁₂ concentration plotted on the left ordinate is represented by the open boxes. Optical density is plotted on the right ordinate with protein the solid dots •—• and sialic acid the open dots O—O.

![Graph](image-url)

**Fig. 4. Distribution of Bound (Nondialyzable) B₁₂* Among Fractions of Chronic Myelogenous Leukemia Seromucoid Separated by Starch Gel Electrophoresis at pH 4.5.**

The concentration of B₁₂* plotted on the left ordinate is represented by the ruled boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots •—•.
bound and was equal to an average of 78 per cent ± 13, range, 57 to 94 per cent.

The distribution of vitamin B₁₂ in a CML serum (Figure 7) is representative of a group of four such sera. As in the normal, the protein concentration of the A-2 to A-6 segments was negligible, while the native vitamin B₁₂ was virtually all found anodally with peak concentrations at A-3 to A-5 segments. The distribution of the native vitamin and of the nondialyzable B₁₂* (Figure 8) is representative of a group of three such sera. The native vitamin had the same anodal
Fig. 7. Native Vitamin B$_2$ Concentration of Protein Fractions of Chronic Myelogenous Leukemic Serum Separated by Starch Gel Electrophoresis at pH 4.5

Vitamin B$_2$ concentration is plotted on the left ordinate and is represented by the open boxes. Optical density of protein is plotted on the right ordinate and is represented by the solid dots $\bullet$. 

distribution as described above. However, in contrast to the normal, a large amount of the added B$_{12}^* (2,200$ to $3,973$ $\mu$g. per ml.) was bound by the A-2 to A-6 segments. The binding of the radiovitamin by these anodal segments was equal to 48 to 78 per cent of the total serum binding of B$_{12}^*$. The per cent saturation of the B$_{12}$BP located at A-2 to A-6 segments ranged from 35 to 75 per cent, as calculated by the method outlined above.

Fig. 8. Distribution of Native Vitamin B$_2$ and of Bound (Non-dialyzable) B$_{12}^*$ Among the Proteins of Chronic Myelogenous Leukemic (CML) Serum Separated by Electrophoresis on Starch Gel at pH 4.5

CML serum and an aliquot of the same serum containing bound (non-dialyzable) B$_{12}^*$ were electrophoresed on the same starch block. The native vitamin shown by the open boxes was measured by Euglena gracilis assay while the bound radiovitamin shown by the ruled boxes was measured by counting the radioactivity in each segment. Note the change in scale of vitamin B$_2$ concentration plotted on the left ordinate from that of Figure 6. The solid dots $\bullet$ refer to protein concentration.
DISCUSSION

A large fraction of the native vitamin $B_{12}$ of normal serum was found in the seromucoid. Cohn Fraction VI, thought to be similar to the seromucoid in composition (19), contained little of the vitamin. In Method VI of Cohn and associates, the major protein fractions of plasma are isolated by a series of precipitations which depend on changes in pH, ionic strength and ethanol concentration (14). Some coprecipitation of the seromucoid routinely occurs when it is prepared from serum by precipitation of the serum proteins with sulfosalicylic or perchloric acid (10), and similarly it may be presumed that coprecipitation of the $B_{12}$BP with each Cohn plasma fraction may account for the absence of selective concentration of the vitamin in Fraction VI.

Since the great bulk of serum proteins have isoelectric points of 4.5 or higher, they either remained at the origin or migrated cathodally at this pH. However, due to the more acidic isoelectric point of the $B_{12}$BP, it migrated anodally with the other proteins of the seromucoid. The native vitamin $B_{12}$ in serum or seromucoid preparations was usually distributed over a relatively wide anodal area of the starch block, with peak concentrations found in a 2 to 3 cm. width. Trailing of the protein and/or diffusion of the $B_{12}$BP during the electrophoretic run may account for the wide distribution of the vitamin and do not necessarily indicate the presence of more than one binding protein. The anodal distribution of vitamin $B_{12}$ was similar to, but not identical with, that of the main protein and sialic acid peaks of the seromucoid or its chief constituent, the acidic $\alpha_1$-glycoprotein (19, 21). Despite their somewhat similar mobility, the $B_{12}$BP is probably not the acidic $\alpha_1$-glycoprotein, since 1) the $B_{12}$BP was not selectively precipitated with Cohn Fraction VI, whereas the acidic $\alpha_1$-glycoprotein has been found only in this fraction (19); and 2) Fahey, McCoy and Goulian fractionated serum on a diethylaminoethyl cellulose ion-exchange column and found that the $B_{12}$BP was eluted off before the acidic $\alpha_1$-glycoprotein (22). A small amount of the native vitamin $B_{12}$ of normal serum (10 per cent) was found scattered among cathodally moving proteins. It is impossible to know whether this represents technical error in the measuring of small amounts of the vitamin or another $B_{12}$BP.

The $B_{12}$BP of CML serum had properties similar to the normal $B_{12}$BP since 1) it was found in the seromucoid, and 2) had anodal mobility when electrophoresed on starch gel at pH 4.5. Mendelsohn, Watkin, Horbett and Fahey found that both the normal and CML $B_{12}$BP behaved similarly when serum was chromatographed on a diethylaminoethyl cellulose ion-exchange column (23). These chemical similarities between the normal and the CML $B_{12}$BP strongly suggest that the increased concentrations of vitamin $B_{12}$ uniformly found in CML serum in relapse is due to an increase in concentration of the normal $B_{12}$BP rather than to an abnormal protein. Despite the tremendous increases in concentration of the $B_{12}$BP in CML sera (amounting to as much as 100-fold in one patient we have studied), only slight increases in the concentration of the total seromucoid fraction have been reported in this disease (24). However, the concentration of $B_{12}$BP in normal serum is probably minute as compared to that of the total seromucoid. Thus, the $B_{12}$BP of a typical normal serum, when completely saturated, can bind approximately 0.65 mg. per ml. If the molecular weight of the $B_{12}$BP is similar to that of the orosomucoid, i.e., 44,000 (25), and if one molecule of the $B_{12}$BP can bind one molecule of vitamin $B_{12}$ (molecular weight, 1,343), the concentration of normal $B_{12}$BP would be equal to 2.20 $\mu$g. per 100 ml. serum. Even a 100-fold increase in concentration of $B_{12}$BP, to 0.22 mg. per 100 ml., would represent only an increase of 0.2 per cent over the normal concentration of the seromucoid, an increase well within the error of the chemical determination.

It would appear that the elevations in seromucoid concentration found in CML are contributed by constituents of the seromucoid other than the $B_{12}$BP.

When additional $B_{12}$* was added to normal serum, the bound (nondialyzable) radiovitamin was almost all associated with proteins found at the origin or cathodal segments, whereas a very small amount of the added $B_{12}$* was bound by the anodally migrating $B_{12}$BP. This suggests that at the levels of vitamin $B_{12}$ found in normal serum, the $B_{12}$BP approaches saturation and, therefore, the addition of further vitamin results...
in the binding of the vitamin to nonacidic proteins. In CML serum on the other hand, there appears to be a commensurate increase of the B12BP in association with the increased level of vitamin B12, so that although the relative saturation of the former remains approximately normal, the absolute binding capacity for added vitamin B12 is greatly increased.

The B12BP has chemical properties similar to those of the acidic α1-glycoprotein and the α2-glycoprotein isolated from Cohn Fraction VI (19, 26). Thus, they are all relatively heat resistant, are all soluble in sulfosalicylic or perchloric acid, are all precipitated by phosphotungstic acid in 2 N HCl and all have acidic isoelectric points as compared with other serum proteins (11, 12, 19, 21, 26, 27). The acidic α1-glycoprotein (19) and the α2-glycoprotein (26) are mucoid glycoproteins, i.e., proteins containing significant amounts of firmly bound hexosamine (28). This would suggest that the B12BP may be a mucoid glycoprotein, although direct confirmation of such a structure must await its isolation in pure form. It is of interest that intrinsic factor (29, 30) and erythropoietin (31) are probably also mucoid glycoproteins. The available evidence suggests that the B12BP is not, however, identical with either of these substances. Thus, intrinsic factor has a differing electrophoretic mobility at pH 8.6 (32) and its binding sites for vitamin B12 are relatively heat labile (33). Recently, we have found a vitamin B12-binding substance in CML urine which resembles that found in CML serum (34). This urinary material, when given orally to patients with pernicious anemia in amounts sufficient to bind an oral dose of B12, had no intrinsic factor activity. Also, increased erythropoietin levels have been reported in such diseases as chronic lymphatic leukemia (35), carcinoma of the cervix (35), secondary polycythemia (36) and hypoplastic anemias (37), whereas vitamin B12 levels are not particularly increased in these conditions.

It seems reasonable to suppose that the B12BP functions as a transport protein for vitamin B12. The seromucoid fraction, representing a readily accessible, easily prepared, and easily concentrated source of the B12BP, should aid in experiments designed to elucidate the role played by this protein in vitamin B12 metabolism.

**SUMMARY**

1. A method for the separation of the vitamin B12-binding protein (B12BP) of normal and chronic myelogenous leukemia serum by electrophoresis on starch gel at pH 4.5 has been described.

2. The B12BP of normal serum has been identified as a constituent of the seromucoid fraction of serum. It is a protein with an electrophoretic mobility at pH 4.5 similar to that of the acidic α1-glycoprotein, the most acidic protein found in serum. However, it was not selectively precipitated with Cohn Fraction VI.

3. The B12BP of chronic myelogenous leukemia serum had properties similar to the normal B12BP, suggesting that the great increase in vitamin B12 levels found in chronic myelogenous leukemia sera results from an increased concentration of the normal B12BP rather than from an abnormal protein.

4. Only a very small fraction of the cobalt60-labeled vitamin B12 bound by normal serum as determined by dialysis was bound to the B12BP. In contrast to the normal, the largest fraction of the cobalt60-labeled vitamin B12 bound by chronic myelogenous leukemia serum was bound by the B12BP.

**REFERENCES**


