Isolation of Vitamin B₁₂-binding Proteins Using Affinity Chromatography

II. PURIFICATION AND PROPERTIES OF A HUMAN GRANULOCYTE VITAMIN B₁₂-BINDING PROTEIN*

(Received for publication, June 26, 1972)

ROBERT H. ALLEN AND PHILIP W. MAJERUS

From the Departments of Internal Medicine and Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

SUMMARY

A vitamin B₁₂-binding protein has been isolated from human granulocytes derived from patients with chronic granulocytic leukemia. Utilizing affinity chromatography as the sole purification technique, the protein was purified 9,860-fold with a yield of over 90% and was homogeneous based on polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein binds 34.9 μg of vitamin B₁₂ per mg of protein and has a single vitamin B₁₂-binding site. The molecular weight determined by sedimentation equilibrium ultracentrifugation was 56,000, whereas that determined by amino acid and carbohydrate analysis was 58,200. The protein contains 33% carbohydrate which accounts for the elevated molecular weight values (121,000 to 138,000) obtained using gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Human granulocytes contain a vitamin B₁₂-binding protein with a molecular weight based upon gel filtration of approximately 115,000 (2, 3). Values of 1.5 (4) and 8.9 (2) ng of vitamin B₁₂-binding activity per 10⁶ leukocytes have been reported, and segmented granulocytes have higher levels than do less mature granulocytes (4) and other leukocytes (2). Simons and Weber have presented indirect evidence that granulocytes synthesize the protein (2). The function of the granulocyte vitamin B₁₂-binding protein is unknown although it has been suggested that granulocytes secrete the protein into plasma to provide one of the major plasma vitamin B₁₂-binding proteins, designated as transcobalamin I. This postulate is supported by: (a) experiments in which intact granulocytes were incubated in vitro and vitamin B₁₂-binding activity was noted to appear in the medium (3). (b) Antibodies to the vitamin B₁₂-binding protein of granulocytes cross-react with transcobalamin I (5). (c) The plasma level of transcobalamin I appears to be directly related to the size of the total body granulocyte pool (6). (d) Carmel and Herbert (7) have reported two brothers who had low levels of vitamin B₁₂-binding protein in their granulocytes and who were also deficient in transcobalamin I.

Vitamin B₁₂-binding proteins are also found in saliva, gastric juice, milk, semen, and cerebrospinal fluid. These proteins also cross-react with antibodies to the granulocyte vitamin B₁₂-binding protein, and this entire group of proteins has been referred to as the “R” proteins by Grässbeck (8). Simons and Weber (2) have suggested that all or part of the vitamin B₁₂-binding protein found in these various body fluids is derived from the granulocytes contained in these secretions. The physiological role of these proteins is unknown; the two patients deficient in these proteins had no apparent abnormality of vitamin B₁₂ metabolism.

Previous attempts to purify the granulocyte vitamin B₁₂-binding protein have been unsuccessful due to the difficulty in collecting sufficient numbers of granulocytes as starting material and to the low yields of vitamin B₁₂-binding protein obtained using conventional purification techniques. We have collected large numbers of granulocytes from untreated patients with chronic granulocytic leukemia and have isolated the granulocyte vitamin B₁₂-binding protein using affinity chromatography as the sole purification procedure. The purification and properties of this protein are described in this report.

EXPERIMENTAL PROCEDURE

Materials

5,5'-Dithiobis(2-nitrobenzoic acid) was obtained from Calbiochem. Dextran grade H was obtained from Pharmacia Corp., Bethlehem, Pa. Human IgG γ-globulin was isolated from normal serum (9) and iodinated using chloramine-T (10). Blue dextran, dinitrophenylalanine, whale myoglobin, rabbit skeletal muscle lactic dehydrogenase, ovalbumin, and porcine chymotrypsin-A were obtained from Sigma Chemical Co. Human transferrin was obtained from Behring Diagnostics. Sodium dodecyl sulfate was obtained from Fisher Chemical Co., and recrystallized by the method of Burgess (11). Other materials were obtained as previously described (12).

* This work was supported by Grants AM 10550 and HE 00022 from the National Institutes of Health, PRA-33 from the American Cancer Society, and Special Research Fellowship AM 51261 from the National Institutes of Health. This work was presented in part at the Meeting of the American Society of Clinical Investigation, Atlantic City, New Jersey, May, 1972 (1).
Methods

Assay of Vitamin B₁₂—Solutions of crystalline vitamin B₁₂ dissolved in water were assayed by measuring the absorbance at 361 nm and 550 nm. Molar extinction coefficients of Ɛ₁₀₀ m = 27,700 and Ɛ₁₀₀ m = 8,680 were used (13). The values for vitamin B₁₂ concentration obtained at each wavelength always agreed within 5% and the average value was used. Solutions containing [⁵⁷Co]vitamin-B₁₂ were assayed in a Packard γ scintillation counter. The vitamin B₁₂ content of granulocyte extracts was assayed by the isotope dilution technique of Lau et al. (14).

Assay for Vitamin B₁₂-binding Ability—Vitamin B₁₂-binding ability was assayed by a modification (12) of the charcoal adsorption method of Gottlieb et al. (15). Samples containing guanidine-HCl were diluted prior to assay, in 0.1 M potassium phosphate, pH 7.5, containing 1 mg/ml of bovine serum albumin. These dilutions were allowed to stand for 30 min at room temperature prior to adding an aliquot of the assay system and the incubation time with [⁵⁷Co]vitamin-B₁₂ was increased to 30 min from the standard incubation time of 15 min.

Protein Assay—Protein was assayed by the method of Lowry et al. (16) using bovine serum albumin as a standard. Preparations of solutions of bovine serum albumin were based on A₄₁₀₀ m = 6.67 (17).

Preparation of vitamin B₁₂ covalently bound to Sepharose was performed as described in the first paper in this series (12).

Polyacrylamide Disc Gel Electrophoresis—Protein solutions were subjected to analytical disc gel electrophoresis at pH 9.5 using the standard 7% analytic system (11). Protein samples containing 10% sucrose were layered on the top of the gels. Gels were stained for protein with Coomassie brilliant blue.

Sodium Dodecyl Sulfate Gel Electrophoresis—Protein samples were adjusted to contain 3% sodium dodecyl sulfate, 0.1 M sodium phosphate, pH 7.4, and 1% 2-mercaptoethanol and were immediately heated for 2 min in a boiling water bath. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, staining and destaining procedures, and molecular weight calculations were carried out as described previously (19, 20).

Absorption Spectra—Absorption and difference spectra were determined at room temperature in a Cary 15 recording spectrophotometer using cuvettes with a 1-cm light path.

Molecular Weight Determination by Gel Filtration—The molecular weight of the granulocyte vitamin B₁₂-binding protein was estimated using a column (2.0 × 90 cm) of Sephadex G-150, fine grade, equilibrated at 4°C with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Samples, in a volume of 6.0 ml of equilibrating buffer, were applied directly to the top of the column. The column flow rate was 15 ml per hour, and 20-ml fractions were usually collected. The gel filtration data are expressed in terms of Kᵥ, the term defined by Laurent and Killander (21):

$$Kᵥ = \frac{Vᵥ - V₀}{V₁ - V₀}$$

Where Vᵥ is the elution volume of a particular protein, V₀ is the void volume of the column, and V₁ is the total available column volume. Blue dextran 2000 was used to determine the value of Vᵥ and was assayed by measuring A₄₁₀₀. Dinitrophenylalanine was used to determine the value of V₁; and was assayed by measuring A₄₁₀₀. The column was calibrated with the following proteins of known molecular weight: myoglobin, 17,800; chymotrypsinogen A, 25,700; ovalbumin, 46,000; transferrin, 89,000; lactic dehydrogenase, 140,000; and human IgG γ globulin 150,000. Vᵥ for protein standards was determined by measuring A₄₁₀₀ except for [⁵⁺¹²] γ-globulin and [⁹³Fe] transferrin that were assayed using a Packard γ scintillation counter. The straight line that expresses the empirically determined relationship between log molecular weight and Kᵥ, was obtained by the method of least mean squares.

Sedimentation Equilibrium—The molecular weight of the vitamin B₁₂-binding protein was measured using the meniscus depletion equilibrium method (22) with ultraviolet optics. Cells were scanned at 360 nm as well as at 361 nm. The partial specific volume was estimated from the amino acid and carbohydrate composition determined on the isolated protein (23, 24). All experiments were performed at 6°C in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Protein samples were dialyzed against this same solution for at least 24 hours. Less than 2% of bound vitamin B₁₂ was removed during the dialysis procedure.

Amino Acid Analysis—Protein solutions for amino acid analysis were dialyzed against distilled water for 24 hours and then lyophilized. Samples (0.1 to 0.3 mg) were hydrolyzed at 105°C for 22 hours in 1 ml of constant boiling HCl in sealed evacuated tubes. Amino acid analyses were performed using a Beckman model 120C amino acid analyzer. The amount of vitamin B₁₂ present was determined by assay of radioactivity in the hydrolysates. Cysteine and methionine were determined after performic acid oxidation (25). Tryptophan was estimated by the method of Edelhoch (26).

Carbohydrate Analysis—Carbohydrate analysis was performed by the method of Clamp et al. (28). Protein samples were subjected to methanolysis, re-N-acetylation, and trimethylsilylation and analyzed by gas-liquid chromatography using an SE-30 liquid phase adsorbed onto Chromosorb Q. A gradient from 140–220°C at 1°C per min was used.

Amino sugars were also determined using the amino acid analyzer as described above.

Isolation of Granulocytes—Granulocytes were obtained from untreated patients with chronic granulocytic leukemia or within several days of the initiation of therapy with busulfan. At the time the granulocytes were collected, peripheral blood contained between 200,000 and 400,000 leukocytes per microliter, and greater than 95% of these cells were in the granulocytic series. Less than 5% of these cells were myeloblasts. Venous blood, 450 ml, was collected under sterile conditions in 67.5 ml of 0.8% citric acid, 2.2% sodium citrate, and 2.4% dextrose per 450 ml of blood in standard blood collection bags and was transferred to a 1000-ml bag that contained 500 ml of 3% NaCl. After standing for 45 min, the supernatant containing greater than 90% of the white blood cells was withdrawn into a separate collecting bag and the remaining red cell sediment was reinfused into the patient. Two 450-ml units of blood were processed daily in this way and up to 4 units were processed from a single patient.

The leucocytes in the dextran supernatant were collected by centrifugation and washes as previously described (29). Final preparations contained less than one erythrocyte per 1000 white cells and less than one platelet per white cell. Greater than 95% of the white cells in the final preparation were in the granulocytic series. The final pellet of white blood cells was suspended in 3 volumes of 0.015 M Tris-HCl, pH 7.4, containing 0.139 M NaCl and was frozen at −70°C and stored in this manner prior to use.
Vitamin B\textsubscript{12} binding assays were performed on sonicates of the final preparation as well as on sonicates of erythrocytes and platelet-rich fractions from the same patients. The results demonstrated that greater than 98\% of the vitamin B\textsubscript{12} binding activity present in the sonicates of the final leukocyte preparation was derived from granulocytes.

Cell suspensions that had been frozen at 70\(^\circ\)C for at least 1 hour were thawed and stirred magnetically for 15 min at 4\(^\circ\)C in an ice bath. Stirring was continued and the cells were disrupted using a Biosonic sonifier equipped with a standard probe. Cells were disrupted for 3 min at 100\% intensity. The sonicate was centrifuged at 20,000 x g for 30 min and the supernatant was removed and combined with the first supernatant. The second supernatant was removed and combined with the first supernatant. Significant amounts of vitamin B\textsubscript{12} binding activity were not obtained when the above procedures were repeated a third time on the second pellet.

Purification of Vitamin B\textsubscript{12} Binding Protein from Human Granulocytes—The starting material in a typical preparation consisted of 500 ml of the supernatant solution obtained after the sonication of 2.7 x 10\(^{11}\) granulocytes from a single donor. The granulocyte extract was filtered with vacuum suction through Celite in a Buchner funnel containing a course sintered glass disc. The Celite washed with 175 ml of 0.0154 M Tris-HCl, pH 7.4, containing 0.139 M NaCl and stirred at 4\(^\circ\)C for 15 min. Sonication was repeated on the suspended pellet followed by centrifugation as described above. The second supernatant was removed and combined with the first supernatant. Significant amounts of vitamin B\textsubscript{12} binding activity were not obtained when the above procedures were repeated a third time on the second pellet.

Renaturation of Granulocyte Vitamin B\textsubscript{12} Binding Protein—As the initial column effluent and each of the column eluates were assayed for vitamin B\textsubscript{12} binding activity and, except for those fractions containing guanidine, were also assayed for protein. The results are summarized in Table I.

The granulocyte vitamin B\textsubscript{12} binding protein has been purified 9860-fold with a recovery of 92.3\% (see Table II). One milligram of protein contained 34.9 \(\mu\)g of vitamin B\textsubscript{12} and had an \(A_{280}:A_{361}\) of 2.17 and an \(A_{335}:A_{361}\) of 2.03. The final material was homogeneous based on polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. Except where indicated, all experiments reported below were performed with this preparation.

**Renaturation of Granulocyte Vitamin B\textsubscript{12} Binding Protein—**

### Table I

**Affinity chromatography of granulocyte vitamin B\textsubscript{12} binding protein**

<table>
<thead>
<tr>
<th>Item</th>
<th>Volume</th>
<th>Vitamin B\textsubscript{12} binding activity</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant of granulocytes</td>
<td>625</td>
<td>35.4</td>
<td>20,400</td>
</tr>
<tr>
<td>Initial vitamin B\textsubscript{12}-Sepharose eluent</td>
<td>660</td>
<td>2.44</td>
<td>1,610</td>
</tr>
<tr>
<td>Other eluates:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 0.1 M pyridine, 0.1 M NaCl</td>
<td>29</td>
<td>0.06</td>
<td>1</td>
</tr>
<tr>
<td>2. 0.1 M glycine, pH 10.0, 1.0 M NaCl, 0.1 M glucose</td>
<td>2,000</td>
<td>0.01</td>
<td>20</td>
</tr>
<tr>
<td>3. 0.1 M potassium phosphate, pH 7.5</td>
<td>80</td>
<td>0.07</td>
<td>6</td>
</tr>
<tr>
<td>4. 5.0 M guanidine HCl, 0.1 M potassium phosphate, pH 7.5</td>
<td>50</td>
<td>5.78</td>
<td>289</td>
</tr>
<tr>
<td>5. 7.5 M guanidine HCl, 0.1 M potassium phosphate, pH 7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Eluted immediately after Eluate 4</td>
<td>12</td>
<td>133</td>
<td>1,600</td>
</tr>
<tr>
<td>b. Eluted 19 hours after 5a</td>
<td>12</td>
<td>382</td>
<td>0.890</td>
</tr>
<tr>
<td>c. Eluted immediately after 5b</td>
<td>12</td>
<td>7.80</td>
<td>88</td>
</tr>
<tr>
<td>d. Eluted 22 hours after 5e</td>
<td>12</td>
<td>40.0</td>
<td>400</td>
</tr>
<tr>
<td>After addition of vitamin B\textsubscript{12} to pooled 5a, 5b, and 5d followed by dialysis and concentration</td>
<td>2.4</td>
<td>8,500*</td>
<td>20,400*</td>
</tr>
</tbody>
</table>

* Based on vitamin B\textsubscript{12} content.
renaturation that occurs when denaturing agents are removed.  Sulphhydryl compounds, or glycerol have increased the amount of renatured from guanidine solutions in the presence of substrates. Changes in pH, protein concentration, salt concentration, or temperature as well as the presence of EDTA, sulphhydryl compounds, or glycerol have increased the amount of renaturation in the absence of vitamin B12. Thus, when a 3-fold excess of vitamin B12 was added prior to dialysis, the protein bound 34.9 μg of vitamin B12 per mg of protein compared to a value of 18 μg of vitamin B12 per mg of protein when the vitamin was added after dialysis.

The increased yield of native protein which results from renaturation in the presence of vitamin B12 is similar to the increased yield of enzyme activity obtained when enzymes are renatured from guanidine solutions in the presence of substrates. For some enzymes, changes in pH, protein concentration, salt concentration, or temperature as well as the presence of EDTA, sulphhydryl compounds, or glycerol have increased the amount of renaturation that occurs when denaturing agents are removed (30-32). Changes in these conditions or the addition of these compounds have not resulted in any significant increase in the renaturation of the granulocyte vitamin B12-binding protein when guanidine is removed in the absence of vitamin B12. Preliminary experiments indicate that sulphhydryl compounds such as 2-mercaptoethanol or dithiothreitol have a deleterious effect on the renaturation reaction.

Removal of Vitamin B12 from Granulocyte Vitamin B12-binding Protein—Vitamin B12 can be removed from the granulocyte vitamin B12-binding protein by dialysis at 22°C against 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. When the granulocyte vitamin B12-binding protein containing vitamin B12 is dialysed against 15 volumes of this dialysate with changes at 24 and 48 hours, greater than 90% of the original vitamin B12 is removed at the end of 72 hours. The granulocyte vitamin B12-binding protein devoid of vitamin B12 can be stored in this guanidine solution at 4°C for at least 4 months without any loss of vitamin B12-binding activity. The ability to remove and replace vitamin B12 was used to increase the specific activity of [14C]vitamin-B12 bound to the granulocyte vitamin B12-binding protein so that studies such as gel filtration could be performed with small quantities of protein.

Polyacrylamide Disc Gel Electrophoresis—When 30 μg of the granulocyte vitamin B12-binding protein-vitamin B12 complex were subjected to polyacrylamide disc gel electrophoresis and stained for protein, a single band was observed as is illustrated in Fig. 1. The unstained gel had a faint red color that was localized to the same area that subsequently stained for protein. Unstained gels were cut to 1-mm sections and the distribution of vitamin B12 was determined by measuring the radioactivity of the individual gel slices. A single peak of radioactivity was observed that coincided with the gel region that stained for protein.

Molecular Weight Determination by Sedimentation Equilibrium—Sedimentation equilibrium experiments were performed with the granulocyte vitamin B12-binding protein-vitamin B12 complex at protein concentrations of 0.058, 0.116, and 0.174 mg per ml. Plots of ln A280 versus R2 and ln A360 versus R2 gave straight lines in all three experiments. The plot of ln A280 versus R2 obtained in the second experiment is shown in Fig. 2.

The values for the slopes of the straight lines obtained by plotting ln absorbance versus R2 were the same when cells were scanned at 280 nm and at 360 nm, indicating correspondence between protein and vitamin B12. No dependence on protein concentration was observed. Using the partial specific volume of 0.710 calculated from the amino acid and carbohydrate analyses (see below), a molecular weight of 55,600 ± 2100 S.D. was obtained for the granulocyte vitamin B12-binding protein-vitamin B12 complex using data from the 280 nm scans. When data from the 360 nm scans were used to calculate the molecular weight, a value of 55,600 ± 5151 S.D. was obtained.

Molecular Weight Determination by Gel Filtration—When 35 μg of the granulocyte vitamin B12-binding protein-vitamin B12 complex (0.04 μg vitamin B12 per μg of vitamin B12) were applied to the calibrated column (2.0 × 90 cm) of Sephadex G-150, a symmetrical peak of radioactivity was observed with an apparent molecular weight of 138,000 (see Fig. 3). This value was grossly different than the value of 55,600 obtained by sedimentation equilibrium. It appears unlikely that protein aggregation accounts for the discrepancy since the same buffer and salt concentration were used for gel filtration and for sedimentation equilibrium and similar temperatures and protein concentrations were employed. Gel filtration experiments were also performed in which aliquots of the granulocyte vitamin B12-binding protein-vitamin B12 complex were removed from the ultracentrifuge cells after sedimentation equilibrium and applied to the Sephadex G-150 column; here the gel filtration molecular weight estimate was also 138,000. The most likely cause for the difference in the molecular weight estimates obtained by sedimentation equilibrium and gel filtration is the 33% carbohydrate content (see below) of the granulocyte vitamin B12-binding protein since proteins that contain large amounts of carbohydrate often give falsely elevated values for molecular weight when determined by gel filtration (33).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—When 25 μg of the granulocyte vitamin B12-binding protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained for protein, a single somewhat diffuse band was observed as shown in Fig. 4. The molecular weight estimate based on the position of this band is 191,000 (20). This value is similar to the value of 138,000 determined by gel filtration (see above) and suggests that the granulocyte vitamin B12-binding protein consists of a single polypeptide chain. The value of 121,000 is probably a falsely elevated estimate as has been reported for other glycoproteins studied in sodium dodecyl sulfate polyacrylamide gel electrophoresis (34, 35).

Amino Acid and Carbohydrate Composition—Except for the
performic acid oxidation analysis and free sulfhydryl and tryptophan determinations, all of the data concerning amino acid and carbohydrate composition were obtained from a second preparation of granulocyte vitamin B$_{12}$-binding protein obtained from a different patient with chronic granulocytic leukemia. The method of purification and yield were the same as detailed above for the first preparation. The second preparation of granulocyte vitamin B$_{12}$-binding protein gave a single band in disc gel electrophoresis, and had an $A_{280}:A_{412}$ ratio of 2.02.

The results of the amino acid and carbohydrate analyses are presented in Table III. Using the molecular weights of the individual amino acids and carbohydrates we determined that the granulocyte vitamin B$_{12}$-binding protein contains approximately 58,200 g of amino acid and carbohydrate per mole of bound vitamin B$_{12}$. This value is similar to the molecular weight value of 56,000 obtained for the granulocyte vitamin B$_{12}$-binding protein by sedimentation equilibrium and suggests that the protein contains a single vitamin B$_{12}$-binding site.

The sulfhydryl group content of the granulocyte vitamin B$_{12}$-binding protein was assayed in 7.5 M guanidine-HCl containing 0.1 M potassium phosphate, pH 7.5. No free sulfhydryl groups (<0.1 residue per mole) were detected, suggesting that any cysteine residues present in this protein are involved in disulfide bonds.

**Adsortion and Difference Spectra**—The spectrum of the granulocyte vitamin B$_{12}$-binding protein-vitamin B$_{12}$ complex in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl is presented in Fig. 5, together with the spectrum of an equal concentration of unbound vitamin B$_{12}$. When vitamin B$_{12}$ is bound to the granulocyte vitamin B$_{12}$-binding protein there appears to be general enhancement of the vitamin B$_{12}$ spectrum above 300 nm since the spectrum of the protein devoid of vitamin B$_{12}$ in 7.5 M guanidine-HCl, 0.05 M potassium phosphate, pH 7.5, is that of a typical protein with insufficient absorption above 300 nm to account for the difference between the two spectra presented in Fig. 5. The spectral maximum of 361 nm for unbound vitamin B$_{12}$ does not change its position when the vitamin is bound to the granulocyte vitamin B$_{12}$-binding protein. The difference spectrum between the granulocyte vitamin B$_{12}$-binding protein-vitamin B$_{12}$ complex and a concentration of unbound vitamin B$_{12}$ containing equal absorption at 361 nm is presented in Fig. 6.

**Discussion**

The method of affinity chromatography described in the first paper in this series (12) has enabled us to isolate the vitamin B$_{12}$-binding protein form granulocytes obtained from patients with chronic granulocytic leukemia. Affinity chromatography was the sole purification technique employed in isolating this protein in homogeneous form. This fact, together with the yield of greater than 90%, demonstrates the utility of this technique. The high yield is particularly important since large numbers of human granulocytes are difficult to obtain and the granulocyte
FIG. 3 (left). Determination of the apparent molecular weight of the isolated human granulocyte vitamin B₁₂-binding protein by gel filtration on a column (2.0 cm X 90 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The proteins used to calibrate the column were: a, IgG γ-globulin; b, lactic dehydrogenase; c, transferrin; d, ovalbumin; e, chymotrypsinogen A; f, myoglobin. X indicates the value for $K_{av}$ obtained for the isolated human granulocyte vitamin B₁₂-binding protein (35 pg) containing 1 pg of $[^{35}S]$vitamin B₁₂.

FIG. 4 (right). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 25 µg of the isolated human granulocyte vitamin B₁₂-binding protein.

FIG. 5. Absorption spectrum of the granulocyte vitamin B₁₂-binding protein. ---, granulocyte vitamin B₁₂-binding protein (174 µg per ml) containing 6.07 µg per ml of vitamin B₁₂. ---, 6.07 µg per ml of unbound vitamin B₁₂. Both spectra were obtained in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The reference cuvette contained the same solution.

Fig. 6. Difference spectrum between the human granulocyte vitamin B₁₂-binding protein-vitamin B₁₂ complex and unbound vitamin B₁₂. The reference cuvette contained 9.12 µg per ml of vitamin B₁₂ in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The second cuvette contained 174 µg per ml of the human granulocyte vitamin B₁₂-binding protein and 6.07 µg per ml of vitamin B₁₂ in the same solution.

vitamin B₁₂-binding protein constitutes only approximately 0.01% of the soluble protein of these cells.

The granulocyte vitamin B₁₂-binding protein binds tightly to vitamin B₁₂-Sepharose and remains bound while the vitamin B₁₂-Sepharose is washed with large volumes of a number of solutions of varying salt concentration ranging in pH from 4.8 to 10.0. Less than 2% of the adsorbed vitamin B₁₂-binding protein is eluted when 15 column volumes of 5.0 M guanidine HCl are passed over the column. Even when 7.5 M guanidine HCl is employed, approximately 41 hours of incubation are required to elute all of the adsorbed granulocyte vitamin B₁₂-binding protein. This resistance to denaturation by guanidine HCl is greater than we have observed for transcobalamin II (36) or for the hog gastric vitamin B₁₂-binding proteins since the bulk of these proteins can be eluted with 5.0 M guanidine HCl and 1 R. H. Allen, unpublished experiments.

TABLE III

<table>
<thead>
<tr>
<th>Item</th>
<th>Residues per mole bound vitamin B₁₂</th>
<th>Item</th>
<th>Residues per mole bound vitamin B₁₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>15</td>
<td>Leucine</td>
<td>42</td>
</tr>
<tr>
<td>Histidine</td>
<td>3</td>
<td>Tyrosine</td>
<td>13</td>
</tr>
<tr>
<td>Arginine</td>
<td>9</td>
<td>Phenylylalanine</td>
<td>12</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>46</td>
<td>Methionine</td>
<td>4*</td>
</tr>
<tr>
<td>Threonine</td>
<td>26</td>
<td>Half-cystine</td>
<td>---</td>
</tr>
<tr>
<td>Serine</td>
<td>36</td>
<td>Tryptophan</td>
<td>10*</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>9</td>
<td>Fucose</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>24</td>
<td>Galactose</td>
<td>24</td>
</tr>
<tr>
<td>Alanine</td>
<td>21</td>
<td>Mannose</td>
<td>14</td>
</tr>
<tr>
<td>Valine</td>
<td>22</td>
<td>N-Acetylgalactosamine</td>
<td>16 (14)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-Acetylglucosamine</td>
<td>34 (31)*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>18</td>
<td>Sialic acid</td>
<td>8</td>
</tr>
</tbody>
</table>

* Determined as methionine sulfone after performic acid oxidation.

a Accurate quantitation was not possible since ninhydrin-positive material was present in the cysteic acid position in the absence of performic acid oxidation. If one assumes that all of the material in this region is cysteic acid then 7 residues were present in the standard analysis and 7 residues were present after performic acid oxidation.

d Determined spectrophotometrically.

Numbers in parentheses were determined from the standard amino acid analysis.
greater than 98% can be eluted by 7.5 M guanidine HCl after only several hours of incubation.

The major disadvantage in eluting the granulocyte vitamin B_{12}-binding protein with guanidine HCl resides in the inability to achieve greater than 50% renaturation (i.e., recovery of vitamin B_{12}-binding activity) when the guanidine is removed by dialysis or dilution in the absence of vitamin B_{12}. The presence of a mixture of denatured and renatured vitamin B_{12}-binding protein would make equilibrium studies of vitamin B_{12} binding difficult to interpret, and because of this we have not attempted to determine the binding constant between vitamin B_{12} and the granulocyte vitamin B_{12}-binding protein. Because of the high degree of affinity that the granulocyte vitamin B_{12}-binding protein shows for vitamin B_{12}-Sepharose, it appears unlikely that conditions can be found which will result in the elution of the granulocyte vitamin B_{12}-binding protein in the absence of significant denaturation of the protein. It might be possible to elute the protein with high concentrations of free vitamin B_{12} but one would still be faced with the problem of removing vitamin B_{12} from the granulocyte vitamin B_{12}-binding protein.

The difference between the molecular weight estimate of 138,000 obtained for the granulocyte vitamin B_{12}-binding protein by gel filtration and the value of approximately 56,000 to 58,000 obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analyses is striking. Discrepancies of this type have been described for proteins with a high degree of affinity that the granulocyte vitamin B_{12}-binding protein shows for vitamin B_{12}-Sepharose, it appears unlikely that conditions can be found which will result in the elution of the granulocyte vitamin B_{12}-binding protein in the absence of significant denaturation of the protein. It might be possible to elute the protein with high concentrations of free vitamin B_{12} but one would still be faced with the problem of removing vitamin B_{12} from the granulocyte vitamin B_{12}-binding protein.

The difference between the molecular weight estimate of 138,000 obtained for the granulocyte vitamin B_{12}-binding protein by gel filtration and the value of approximately 56,000 to 58,000 obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analyses is striking. Discrepancies of this type have been described for proteins with a high degree of affinity that the granulocyte vitamin B_{12}-binding protein shows for vitamin B_{12}-Sepharose, it appears unlikely that conditions can be found which will result in the elution of the granulocyte vitamin B_{12}-binding protein in the absence of significant denaturation of the protein. It might be possible to elute the protein with high concentrations of free vitamin B_{12} but one would still be faced with the problem of removing vitamin B_{12} from the granulocyte vitamin B_{12}-binding protein.

The difference between the molecular weight estimate of 138,000 obtained for the granulocyte vitamin B_{12}-binding protein by gel filtration and the value of approximately 56,000 to 58,000 obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analyses is striking. Discrepancies of this type have been described for proteins with a high degree of affinity that the granulocyte vitamin B_{12}-binding protein shows for vitamin B_{12}-Sepharose, it appears unlikely that conditions can be found which will result in the elution of the granulocyte vitamin B_{12}-binding protein in the absence of significant denaturation of the protein. It might be possible to elute the protein with high concentrations of free vitamin B_{12} but one would still be faced with the problem of removing vitamin B_{12} from the granulocyte vitamin B_{12}-binding protein.

Acknowledgments—We thank Dr. David Alpers for performing the carbohydrate analyses using gas liquid chromatography. We also thank Carol Meldman and Carmelita Lowry for their assistance. Irene Dorner assisted in collections of granulocytes from patients with chronic granulocytic leukemia.
Isolation of Vitamin B₁₂-binding Proteins Using Affinity Chromatography: II. PURIFICATION AND PROPERTIES OF A HUMAN GRANULOCYTE VITAMIN B₁₂-BINDING PROTEIN

Robert H. Allen and Philip W. Majerus


Access the most updated version of this article at http://www.jbc.org/content/247/23/7702

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/23/7702.full.html#ref-list-1