Isolation of Vitamin B₁₂-binding Proteins
Using Affinity Chromatography

III. PURIFICATION AND PROPERTIES OF HUMAN PLASMA TRANSCOBALAMIN II*

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

Transcobalamin II has been isolated from Cohn Fraction III derived from 1,400 liters of pooled human plasma, using affinity chromatography on vitamin B₁₂-Sepharose and several conventional purification techniques. The final preparation was purified 2 million-fold relative to human plasma with a yield of 12.8% and was homogeneous based on polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and chromatography on Sephadex G-150. Transcobalamin II binds 28.6 μg of vitamin B₁₂ per mg of protein and contains one vitamin B₁₂-binding site per 59,500 g of protein as determined by amino acid analysis. The molecular weight determined by sedimentation equilibrium ultracentrifugation was 53,900 and by gel filtration on Sephadex G-150 was 60,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis disclosed two peptides with molecular weight values of 38,000 and 25,000 which suggests that transcobalamin II contains 2 subunits.

When vitamin B₁₂ binds to transcobalamin II there is a shift in the peak of vitamin B₁₂ absorption from 361 nm to 304 nm. Analysis of transcobalamin II for carbohydrate content using gas-liquid chromatography and amino sugar analysis by the amino acid analyzer suggest that this trace plasma protein is not a glycoprotein.

Additional studies concerning the plasma vitamin B₁₂-binding proteins have been limited by the fact that the vitamin B₁₂-binding capacity of human plasma is less than 2 μg per liter (3, 4). Using the molecular weight values obtained by gel filtration and assuming one vitamin B₁₂-binding site per molecule of vitamin B₁₂-binding protein, 1 liter of human plasma contains less than 100 μg of either transcobalamin I or transcobalamin II. Purification in excess of a million-fold would be required to achieve homogeneity for either of the proteins, and...
this has been beyond the limits of conventional purification techniques.

Using affinity chromatography in addition to ion exchange chromatography and gel filtration, we have succeeded in isolating transcobalamin II. This report is concerned with the purification and physical properties of this protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cohn Fraction III was obtained from the American Red Cross National Fractionation Center. Other materials were obtained as described in the first two papers in this series (13, 14).

**Methods**

Vitamin B₁₂-binding assays were performed using a modification (13, 14) of the method of Gottlieb et al. (15). Solutions containing radioactive and nonradioactive vitamin B₁₂ were assayed as described in the first paper in this series (13). The isolation of monocarboxylic acid derivatives of vitamin B₁₂ and their covalent attachment to 3,3’-diaminopropylamine-substituted Sepharose using a carbodiimide was performed as described in the first paper in this series (13). The content of covalently bound vitamin B₁₂ was 0.68 μmole per ml of packed Sepharose.

Protein assays, polyacrylamide disc gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, sedimentation equilibrium ultracentrifugation, molecular weight determinations by gel filtration, amino acid analysis, assay of sulphydryl group content, carbohydrate analysis, and absorption and difference spectra were all performed as described in the second paper in this series (14).

**Purification of Transcobalamin II**

**Step 1: Cohn Fraction III of Human Plasma—Transcobalamin II** was purified starting with Cohn Fraction III of pooled human plasma. All procedures were performed at 4°C. Each lot of Cohn Fraction III (72 kg) was derived from approximately 3000 liters of pooled human plasma. A typical purification using 34 kg of this material is described below. The frozen material was chopped with an ice pick into pieces weighing less than 500 g and 8.5 kg of these frozen pieces of Cohn Fraction III were collected from the 8 liters of suspension remaining in each container. This suspension was approximately 5.8.

**Step 2: Cm-Sephadex—Sixty-three grams of dry, unprocessed Cm-Sephadex-U-50 were added to each container and stirring was continued for an additional 4 hours. After the addition of the Cm-Sephadex, the pH of the suspension rose to 5.9. Stirring was stopped and the Cm-Sephadex was allowed to settle overnight. The upper 85 liters of each container were next removed through a siphon and discarded. The Cm-Sephadex was collected from the 8 liters of suspension remaining in each container by suction filtration using a Buchner funnel and 24-cm diameter circles of S & S filter paper No. 885. Approximately 2 kg of Cm-Sephadex, wet weight, were recovered from each of the large plastic containers, and the Cm-Sephadex from each container was suspended in 4 liters of the original Cohn Fraction III suspension solution. After stirring for 5 min each suspension of Cm-Sephadex was again collected by suction filtration. Each batch of Cm-Sephadex was then suspended in 2 liters of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M sodium chloride and stirred with a magnetic stirrer for 30 min. Each suspension was suction-filtered on a Buchner funnel containing a 24.5-cm diameter circle of S & S glass wool No. 24 on top of a 24-cm diameter circle of S & S filter paper No. 885. The filter cake was then washed directly on the Buchner funnel with an additional 2 liters of the same eluting solution. A combined total of 19,700 ml of this elution filtrate was obtained and contained 78% of the vitamin B₁₂-binding activity present in the initial Cohn Fraction III suspension. The elution filtrate contained only a faint turbidity and was used directly for affinity chromatography on vitamin B₁₂-Sephadex.

**Step 3: Affinity Chromatography on Vitamin B₁₂-Sepharose—A column 2.5 cm in diameter and 2 cm tall of vitamin B₁₂-Sepharose was prepared and washed with 100 ml of 0.1 M glycine-NaOH, pH 10.0, followed by 100 ml of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. This procedure served to remove traces of vitamin B₁₂ which had become hydrolyzed from covalent linkage to Sepharose. The entire 1.0 M NaCl elution filtrate from the previous Cm-Sephadex batch step was then applied to the column of vitamin B₁₂-Sepharose with a gravity head of approximately 250 cm of water. The flow rate was approximately 500 ml per hour. Only 7.7% of the vitamin B₁₂-binding activity applied to the vitamin B₁₂-Sepharose column was recovered in the total effluent. Small aliquots of the effluent were collected directly from the vitamin B₁₂-Sepharose column at various times during the sample application. These aliquots were also assayed for vitamin B₁₂-binding activity. They in dicated that early in the sample application greater than 99% of the vitamin B₁₂-binding activity was adsorbed to the vitamin B₁₂-Sepharose and that this level of adsorption had fallen to 90% near the end of the sample application. After the entire sample had been applied, the column was then washed with distilled water volumes of a variety of solutions in the following order. Wash 1: 100 ml of 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. Wash 2: 500 ml of 0.1 M potassium phosphate, pH 7.5. Wash 3: 1930 ml of 0.1 M glycine-NaOH, pH 10.0, containing 1.0 M NaCl and 0.1 M glucose. Wash 4: 300 ml of 0.1 M potassium phosphate, pH 5.8, containing 1.0 M NaCl. Wash 5: 150 ml of H₂O. Wash 6: 275 ml of 0.1 M potassium phosphate, pH 7.5. Wash 7: 100 ml of 0.1 M potassium phosphate, pH 7.5, containing 0.75 M guanidine HCl. The flow rate during the first six column washes was 200 ml per hour and that of Wash 7 was 100 ml per hour. The effluent from each wash was collected separately. At the completion of Wash 7, the flow rate was decreased to 20 ml per hour and a solution of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl was applied. The first 43.0 ml of column effluent were collected in their entirety and were designated as column Wash 8a. The next 5.5 ml effluent from Wash 8 was collected separately and designated as column Wash 8b. At this point the column was clamped and allowed to stand for 18 hours. At the end of this time the column was unclamped and the first 13.0 ml of effluent were collected and were designated as column Wash 8c. Each of the column effluents mentioned above was assayed for vitamin B₁₂-binding activity and, except for those fractions containing guanidine, was also assayed for protein content. The results are presented in Table I. Eluate 8a from vitamin B₁₂-Sepharose affinity chromatography was mixed with [³⁸Co]vitamin-B₁₂ (1320 μg, 0.0034 μCi per μg) in a final volume of 44 ml. This mixture was dialyzed against 6 liters of 0.1 M Tris-HCl, pH 8.9, contain-
Further elutions of vitamin B₁₂-Sepharose:

After addition of 1,320 μg of vitamin B₁₂, the transcobalamin II-vitamin B₁₂ fraction was centrifuged at 50,000 × g for 30 min to remove denatured protein prior to chromatography on DEAE-cellulose.

**Step 4: Chromatography on DEAE-Cellulose**—A column (0.9 × 12 cm) of DEAE (Whaterman DE 52) equilibrated with 0.1 M Tris-HCl, pH 8.9, was first washed with 60 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.0111 μg of [⁵⁷Co]vitamin-B₁₂ per ml (0.0034 μCi per μg) before the transcobalamin II-vitamin B₁₂ fraction from *Step 3* was applied to the column at a flow rate of 25 ml per hour. The column was washed with 10 ml of the 100 mM Tris-vitamin-B₁₂ containing equilibrating solution and then eluted with a linear gradient in which the mixing chamber contained 225 ml of 0.1 M Tris-HCl, pH 8.9, and the reservoir contained 225 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.5 M NaCl. All of these eluting solutions contained [⁵⁷Co]vitamin-B₁₂ as described above. Fractions were assayed for A₃₅₀, vitamin B₁₂ content, and conductivity. The results are presented in Fig. 1. Fractions 50 through 65 were pooled.

**Step 5: Chromatography on 3,3'-Diaminodipropylamine-substituted Sepharose**—A column (0.9 × 6 cm) of 3,3'-diaminodipropylamine-substituted Sepharose was equilibrated with 100 ml of 0.1 M Tris-HCl, pH 8.9, containing 0.2 M NaCl. The DEAE-cellulose pooled fractions 50 to 65 of transcobalamin II-vitamin B₁₂ were applied to the column at a flow rate of 50 ml per hour and the column was eluted with this same buffer. The first 82 ml of effluent from the column contained greater than 99% of the transcobalamin II-vitamin B₁₂ applied. The transcobalamin II-vitamin B₁₂ solution was adjusted to contain 0.75 M NaCl and was then concentrated to approximately 1 ml using an Amicon ultrafiltrator equipped with a Diaflo UM-10 membrane. Despite stirring during the concentration procedure, a red film was observed on the Diaflo membrane at the completion of the concentration procedure. The Amicon concentrate was removed and the concentrating vessel was rinsed repeatedly with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl until the red film on the membrane went into solution. The final concentrate was slightly turbid and this precipitate was removed by centrifugation at 10,000 × g for 10 min. Approximately 4% of the total vitamin B₁₂ present was present in the small pink precipitate, with the remaining 96% being present in the 0.9 ml of red supernatant solution. This supernatant solution was immediately subjected to chromatography on Sephadex G-150.

**Step 6: Chromatography on Sephadex G-150**—A column (2.0 × 90 cm) of Sephadex G-150, fine grade, was equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl and [⁵⁷Co]vitamin-B₁₂ (0.0111 μg per ml, 0.0034 μCi per μg). The transcobalamin II-vitamin B₁₂ fraction from the preceding step was applied directly to the top of the column and the column was eluted with the equilibrating solution at a flow rate of 20 ml per hour. Fractions of 3.3 ml were collected and assayed for vitamin B₁₂ content and for absorption at 280 nm (Fig. 2). Fractions 51 to 64 were pooled and concentrated using an Amicon ultrafiltrator as described in *Step 5*. A red film also observed on the Diaflo membrane at the end of this concentration procedure was dissolved as described in *Step 5*. Less than 1% of the vitamin B₁₂ placed in the Amicon ultrafiltrator passed through the UM-10 membrane. The Amicon concentrate and the rinses were combined, centrifuged at 10,000 × g for 10 min, and the red supernatant decanted. A small dark red pellet containing 2% of the total vitamin B₁₂ present was discarded. The red supernatant, containing 98% of the vitamin B₁₂, was divided into 1.5-ml aliquots, quick-frozen in a Dry Ice-acetone bath, and stored at −70°C. A summary of the purification procedure is presented in Table II.

### RESULTS

Original attempts to purify transcobalamin II by passing plasma directly over vitamin B₁₂-Sepharose columns were unsuccessful because of the viscous nature of plasma and the fact

![Table I](http://www.jbc.org/)

**Affinity chromatography of transcobalamin II**

| Item                                      | Volume (ml) | Vitamin B₁₂-binding activity (μg/ml) | Protein (mg/ml) | Flow rate (ml/hr) |
|-------------------------------------------|-------------|-------------------------------------|----------------|-------------------|
| Initial effluent from vitamin B₁₂-Sepharose | 19,700      | 22.8                                | 417,000        | 700,000          |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 500         | 0.74                                | 370            | 100              |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 1,950       | 2.12                                | 4,130          | 1,570            |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 300         | 0.62                                | 186            | 3                |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 150         | 0.38                                | 57             | 0                |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 275         | 0.03                                | 8              | 0                |
| Eluate 8a after addition of 1,320 μg of vitamin B₁₂ | 100         | 0.44                                | 44             | 100              |

*Vitamin B₁₂ content.*
Fig. 1. Elution pattern (purification step 4) of the transcobalamin II-vitamin B₁₂ complex after chromatography on a column (0.9 × 12 cm) of DEAE-cellulose. The column was equilibrated with 0.1 M Tris-HCl, pH 8.9, prior to sample application and was eluted with a linear gradient in which the mixing chamber contained 225 ml of 0.1 M Tris-HCl, pH 8.9, and the reservoir contained 225 ml of Tris-HCl, pH 8.9, 0.5 M NaCl. ○, A₁₂₅; ●, vitamin B₁₂; ■, conductivity. The arrow indicates the point at which the gradient was begun.

Fig. 2 (left). Elution profile (purification step 6) of the transcobalamin II-vitamin B₁₂ complex on a column (2.0 × 90 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl and 0.0111 μg of [³⁵S]vitamin-B₁₂ (0.0034 μCi per μg) per ml. ○, A₁₂₅; ●, vitamin B₁₂; ▲, specific activity. V₀ and Vₐ were determined with blue dextran 2000 and dinitrophenylalanine, respectively, during eight other gel filtration experiments under the same conditions as above with less than 1% variation in their position. On the elution profile shown here the transcobalamin II-vitamin B₁₂ complex has an apparent molecular weight of 60,000 as indicated (see Fig. 6). The expected elution positions for 38,000 and 25,000 molecular weight material are also indicated.

Fig. 3 (right). Polyacrylamide disc gel electrophoresis of 30 μg of transcobalamin II containing 1.05 μg of bound vitamin B₁₂.
that precipitates form during chromatography causing slow column flow rates.

Cohn Fraction III, supplied as a frozen wet paste in 72-kg lots derived from 3000 liters of pooled human plasma, was used as the starting material. We have tested five separate lots of Cohn Fraction III and have observed vitamin B\textsubscript{12}-binding activities ranging from 12 to 18 ng of vitamin B\textsubscript{12} bound per g of frozen wet paste. No loss of activity has been noted after storage of Cohn Fraction III at \(-20^\circ\) for several months. Based on the binding of the vitamin B\textsubscript{12}-binding protein in Cohn Fraction III to Cm-Sephadex as well as its elution profile on Sephadex G-150, we conclude that greater than 95% of the vitamin B\textsubscript{12}-binding activity in Cohn Fraction III is attributable to transcobalamin II (2). Assuming that pooled human plasma contains 1 ng per ml of transcobalamin II-vitamin B\textsubscript{12} binding activity, 27% to 40% of the transcobalamin II present in plasma is recovered in Cohn Fraction III.

As shown in Table II transcobalamin II is partially purified by batch chromatography on Cm-Sephadex before it is further purified by affinity chromatography on vitamin B\textsubscript{12}-Sepharose. The Cm-Sephadex step results in a 20-fold reduction in volume and a 5-fold purification, but its major advantage is that transcobalamin II is obtained in a solution that is capable of passing over a column of vitamin B\textsubscript{12}-Sepharose without eluting the column. Initial attempts to suspend Cohn Fraction III in various buffers followed by centrifugation failed to produce solutions that were suitable for direct application to vitamin B\textsubscript{12}-Sepharose because of protein precipitation.

The composition of the solution used to suspend Cohn Fraction III (0.02 M sodium phosphate, pH 5.7, 0.1 M NaCl) is important, since at lower concentrations of NaCl transcobalamin II does not go into solution while at higher concentrations it does not bind to Cm-Sephadex. Transcobalamin II was eluted from Cm-Sephadex with 0.1 M sodium phosphate, pH 5.8, containing 1.0 M NaCl. Transcobalamin II can also be eluted from Cm-Sephadex with 0.1 M sodium phosphate at pH values greater than 8.0, but this eluate precipitates within hours of the elution process which makes affinity chromatography on vitamin B\textsubscript{12}-Sepharose impossible. Transcobalamin II is relatively unstable after elution from Cm-Sephadex since about 10% of the vitamin B\textsubscript{12}-binding activity is lost per 24 hours at 4°C.

No attempt has been made to determine the amount of transcobalamin II already containing bound vitamin B\textsubscript{12} that is present in Cohn Fraction III, nor have we analyzed the fate of this complex during the early purification steps. Based on our binding (see below) that transcobalamin II has one vitamin B\textsubscript{12}-binding site per molecule, we would not expect that transcobalamin II already containing vitamin B\textsubscript{12} would be adsorbed by the vitamin B\textsubscript{12}-Sepharose column.

Affinity chromatography on vitamin B\textsubscript{12}-Sepharose results in a 24,000-fold purification of transcobalamin II, but approximately 50% contaminating protein is still present after this purification step. This result is in contrast to the purification of the granulocyte vitamin B\textsubscript{12}-binding protein (14) where no detectable contaminating protein is present after affinity chromatography. The most likely reason for this difference resides in the fact that 98% of the granulocyte vitamin B\textsubscript{12}-binding protein remained adsorbed to vitamin B\textsubscript{12}-Sepharose when the column was washed with 5.0 M guanidine HCl while significant amounts of transcobalamin II are eluted with 5.0 M guanidine HCl and this washing procedure could not be employed for transcobalamin II. The comparative ease of elution of transcobalamin II is also demonstrated by the fact that only several hours of incubation with 7.5 M guanidine are required for elution (see Table I) while 41 hours are required for the granulocyte vitamin B\textsubscript{12}-binding protein (14).

Transcobalamin II has been purified 2 million-fold relative to plasma with a recovery of 12.8% (Table II). The final preparation is homogeneous based on results of disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, gel filtration on Sephadex G-150, and the ratio of total amino acid content to bound vitamin B\textsubscript{12}. Based on the pooled Sephadex G-150 fractions, 1 mg of protein contains 28.6 µg of bound vitamin and has an A\textsubscript{280}/A\textsubscript{260} of 1.5 and an A\textsubscript{250}/A\textsubscript{260} of 0.74. The ratio of A\textsubscript{280}/A\textsubscript{260} is 2.04.

Solubility of Transcobalamin II-Vitamin B\textsubscript{12} Complex—Transcobalamin II saturated with vitamin B\textsubscript{12} precipitates under a variety of conditions, e.g. dialysis of transcobalamin II-vitamin B\textsubscript{12} (0.1 mg of protein per ml) against H\textsubscript{2}O or 0.1 M sodium acetate pH 5.5. Detailed studies concerning transcobalamin II solubility have not been conducted but the precipitation of the transcobalamin II-vitamin B\textsubscript{12} complex appears favored by high protein concentration, low pH values, and decreased ionic strength. The transcobalamin II-vitamin B\textsubscript{12} complex is soluble in 0.05 M potassium phosphate containing 0.75 M NaCl at protein concentrations as high as 1 mg per ml. Solutions of this
composition were utilized for storage of the protein as well as for performing many of the physical studies outlined below.

Removal of Vitamin $B_{12}$ from Transcobalamin II—Vitamin $B_{12}$ can be removed from transcobalamin II by dialysis at room temperature against 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. When transcobalamin II containing vitamin $B_{12}$ is dialysed against 15 volumes of this solution with changes at 24 and 48 hours, greater than 99% of the original bound vitamin $B_{12}$ is removed in 72 hours. Transcobalamin II devoid of vitamin $B_{12}$ can be stored in this guanidine solution at 4°C for at least 3 months without significant loss of vitamin $B_{12}$-binding activity as assayed by adding a 3-fold excess of vitamin $B_{12}$ (containing radioactive vitamin $B_{12}$) to the transcobalamin II guanidine solution, followed by removal of guanidine and unbound vitamin $B_{12}$ by dialysis against 0.1 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The ability to remove and then replace the original bound vitamin $B_{12}$ was used to increase the content of $^{32}K$vitamin-$B_{12}$ so that certain studies, such as gel filtration, could be performed with small quantities of protein.

Factors Influencing Renaturation of Transcobalamin II—The renaturation (i.e. vitamin $B_{12}$-binding ability) of transcobalamin II is greater when guanidine is removed by dialysis in the presence of a 3-fold excess of vitamin $B_{12}$ than when aliquots of the protein in guanidine are dialyzed against 1:5:5000 and assayed for vitamin $B_{12}$-binding activity directly. This is illustrated in Table I where the initial 7.5 M guanidine HCl eluate from vitamin $B_{12}$-Sepharose bound 353,000 ng of vitamin $B_{12}$ by the former method and only 198,000 ng by the latter method. Similar results are obtained using the final preparation of transcobalamin II. Thus, when a 3-fold excess of vitamin $B_{12}$ is added prior to dialysis, transcobalamin II binds 27-30 μg vitamin $B_{12}$ per mg of protein compared to a value of 14 to 18 μg of vitamin $B_{12}$ per mg of protein when the vitamin is added after dialysis or after a 5000 to 75,000 dilution of a solution containing the protein and guanidine. These results indicate that the presence of the vitamin is an important factor in the renaturation process. A similarly increased yield of native protein after renaturation in the presence of vitamin $B_{12}$ was also observed for the granulocyte vitamin $B_{12}$-binding protein (14).

In other studies variation in protein concentration, temperature, pH, and salt concentrations as well as the addition of EDTA, sulfhydryl compounds, and glycerol have not resulted in any significant increase in the renaturation (i.e. vitamin $B_{12}$-binding activity) of transcobalamin II when guanidine is removed in the absence of vitamin $B_{12}$. The presence of 0.02 M 2-mercaptoethanol and dithiothreitol both cause a marked decrease in the degree of transcobalamin II renaturation.

Polyacrylamide Disc Gel Electrophoresis—When 30 μg of the transcobalamin II-vitamin $B_{12}$ complex was subjected to polyacrylamide disc gel electrophoresis and stained for protein the pattern presented in Fig. 3 was obtained. Unstained gels had a faint red color that was localized to the entire region of the gel that stained for protein. Unstained gels were cut into 1-mm sections and the distribution of vitamin $B_{12}$ was determined by measuring the radioactivity of the individual gel slices. A single broad peak of radioactivity was observed that coincided with the gel region that stained for protein. The reason for the failure to obtain a sharper band of either protein or vitamin $B_{12}$ has not been determined but may be related to the limited solubility (see above) of the transcobalamin II-vitamin $B_{12}$ complex since high protein concentrations are achieved during the stacking period of disc gel electrophoresis.

Molecular Weight Determination by Sedimentation Equilibrium—Sedimentation equilibrium experiments were performed with the transcobalamin II-vitamin $B_{12}$ complex at protein concentrations of 0.008, 0.136, and 0.204 mg per ml in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Plots of In $A_{280}$ versus $R^2$ and In $A_{360}$ versus $R^2$ gave straight lines in all three experiments. The plot of In $A_{280}$ versus $R^2$ obtained at 0.136 mg of protein per ml is shown in Fig. 4. The values for the slopes of the straight lines obtained by plotting In absorbance versus $R^2$ were the same when cells were scanned at 280 nm and 360 nm indicating correspondence between protein and vitamin $B_{12}$. No dependence on protein concentration was observed. Using the partial specific volume of 0.74 calculated from the amino acid analysis (see below) a molecular weight of 53,900 ± 2,500 S.D. was obtained for the transcobalamin II-vitamin $B_{12}$ complex using the data from the 360 nm scans. When data from one of the 360 nm scans were used to calculate the molecular weight, a value of 52,800 was obtained.

Amino Acid Analysis of Transcobalamin II—The amino acid composition of transcobalamin II is presented in Table III. When sulfhydryl groups were assayed in 1.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5, no sulfhydryl residues were detected (<0.1 residue per mole of transcobalamin II). This finding indicates that any cysteine residues in transcobalamin II are involved in disulfide bands.

Based on the molecular weights of the individual amino acids determined, transcobalamin II contains 59,500 g of amino acid per mole of bound vitamin $B_{12}$. This value is close to the respective molecular weights of 53,900 and 60,000 determined for the transcobalamin II-vitamin $B_{12}$ complex by sedimentation equilibrium ultracentrifugation (see above) and gel filtration on Sephadex G-50 (see below). The agreement among these studies indicates that transcobalamin II contains a single vitamin $B_{12}$-binding site and that the final preparation of transcobalamin II is devoid of major contamination by denatured transcobalamin II or other proteins.

Carbohydrate Analysis—No carbohydrate residues were detected in the final preparation of transcobalamin II by gas-liquid chromatography and no amino sugar residues were detected on the amino acid analyzer. The amount of protein
trophoresis has suggested that transcobalamin II consists of 2 molecular weight of 60,000. Sodium dodecyl sulfate gel electrophoresis of these experiments, a single symmetrical peak of vitamin B12-vitamin B12 complex from this experiment was 60,000. \( K_a \) indicates the value for \( K_a \) obtained when 40 \( pg \) of the final preparation of transcobalamin II was applied to the same column of Sephadex G-150 in the presence and absence of \([^{57}Co\]vitamin-B12. An apparent molecular weight of 38,000 was obtained in both of these experiments. (See text for additional details and comment.)

Other gel filtration experiments were performed on the same Sephadex G-150 column using samples of transcobalamin II which were 250-fold less concentrated with respect to protein than in the experiment described above. Eighty micrograms of the isolated protein were dialyzed against 7.5 \( m \) guanidine HCl to remove greater than 99% of the bound vitamin B12. Half of this protein solution was then dialyzed against 300 volumes of 0.05 \( m \) potassium phosphate, pH 7.5, containing 0.75 \( m \) NaCl for 72 hours with changes at 24 and 48 hours. The other half was dialyzed in an identical manner except that 3.4 \( \mu g \) of \([^{57}Co\]vitamin-B12 were added to the protein-guanidine solution prior to dialysis. Each of the two dialyzed protein solutions was adjusted to a volume of 6.0 ml containing 10 \( \mu g \) of blue dextran 2000 and 2 \( \mu g \) of dimethylphenyl alamine and applied separately to the Sephadex G-150 column. In both of these experiments, a single symmetrical peak of vitamin B12-binding activity (or \([^{57}Co\]vitamin-B12) was observed at an elution position corresponding to a molecular weight of 38,000 (see Fig. 6). These two results suggest the possibility that the transcobalamin II subunits were not associated under the conditions in which these experiments were performed and that the 38,000 molecular weight subunit contains the binding site for vitamin B12. It is also possible that transcobalamin II interacts with Sephadex at low protein concentrations with a resulting retardation of the protein.

**Table III**

Amino acid composition of transcobalamin II

| Amino acid | Residues of amino acid per mole of bound vitamin B12 | Amino acid | Residues of amino acid per mole of bound vitamin B12 |
|------------|-----------------------------------------------|------------|-----------------------------------------------|
| Lysine     | 24                                            | Alanine    | 42                                            |
| Histidine  | 20                                            | Valine     | 23                                            |
| Arginine   | 25                                            | Isoleucine | 16                                            |
| Aspartic acid | 37                           | Leucine    | 92                                            |
| Threonine  | 27                                            | Tyrosine   | 14                                            |
| Serine     | 37                                            | Phenylalanine | 14                                    |
| Glutamic acid | 71                  | Methionine | 10\(^a\)                                     |
| Proline    | 27                                            | Half-cystine | 6\(^b\)                                     |
| Glycine    | 43                                            | Tryptophan | 6\(^b\)                                     |

\(^a\) Determined as methionine sulfone after performic acid oxidation.

\(^b\) 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 9 residues were present in the standard analysis and 13 residues were present after performic acid oxidation.

\(^c\) Determined spectrophotometrically.

**Fig. 5.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 30 \( pg \) of transcobalamin II. Electrophoresis was performed for 7.5 hours, and at the end of this time the tracking dye was located 3 cm from the bottom (left) of the 20-cm gel. The mobility of the two protein bands indicates molecular weights of 38,000 and 25,000.

**Fig. 6.** Determination of the apparent molecular weight of transcobalamin II by gel filtration on a column (2.0 cm \( \times \) 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 \( \gamma \)-globulin; b, lactic dehydrogenase; c, transferring \( \alpha \), ovalbumin; e, chymotrypsinogen; f, myoglobin. X indicates the value for \( K_a \) obtained for the transcobalamin II-vitamin B12 complex during the final purification (see Fig. 2). The apparent molecular weight obtained for the transcobalamin II-vitamin B12 complex from this experiment was 60,000. \( \phi \) indicates the value of \( K_a \) obtained when 40 \( pg \) of the final preparation of transcobalamin II was applied to the same column of Sephadex G-150 in the presence and absence of \([^{57}Co\]vitamin-B12. An apparent molecular weight of 38,000 was obtained in both of these experiments. (See text for additional details and comment.)

**TABLE III**

| Amino acid | Residues of amino acid per mole of bound vitamin B12 |
|------------|-----------------------------------------------|
| Lysine     | 24                                            |
| Histidine  | 20                                            |
| Arginine   | 25                                            |
| Aspartic acid | 37                           |
| Threonine  | 27                                            |
| Serine     | 37                                            |
| Glutamic acid | 71                  |
| Proline    | 27                                            |
| Glycine    | 43                                            |

| Residues of amino acid per mole of bound vitamin B12 | Residues of amino acid per mole of bound vitamin B12 |
|-----------------------------------------------------|-----------------------------------------------------|
| Lysine                                              | 24                                                  |
| Histidine                                           | 20                                                  |
| Arginine                                           | 25                                                  |
| Aspartic acid                                      | 37                                                  |
| Threonine                                          | 27                                                  |
| Serine                                             | 37                                                  |
| Glutamic acid                                      | 71                                                  |
| Proline                                            | 27                                                  |
| Glycine                                            | 43                                                  |
Absorption and Difference Spectra—The spectrum of the transcobalamin II-vitamin B12 complex is presented in Fig. 7 together with the spectrum of an equal concentration of unbound vitamin B12. When vitamin B12 is bound to transcobalamin II there appears to be a general enhancement of the vitamin B12 spectrum above 300 nm since the spectrum of transcobalamin II devoid of vitamin B12 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. 7. When vitamin B12 binds to transcobalamin II, there is a shift in the 361 nm spectral maximum for unbound vitamin B12 to 364 nm for the transcobalamin II-vitamin B12 complex. The difference spectrum between the transcobalamin II-vitamin B12 complex and a concentration of unbound vitamin B12 containing equal absorption at 361 nm is presented in Fig. 8.

**FIG. 7.** Absorption spectra of the transcobalamin II-vitamin B12 complex and of unbound vitamin B12. Spectra were obtained in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The reference cuvette contained the same solution. --- transcobalamin II (400 µg per ml) containing 11.7 µg per ml of bound vitamin B12; --- vitamin B12 (11.7 µg per ml).

**FIG. 8.** Difference spectrum between the transcobalamin II-vitamin B12 complex and unbound vitamin B12 in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The reference cuvette contained 17.9 µg per ml of unbound vitamin B12 and the second cuvette contained 11.7 µg per ml of vitamin B12 and 400 µg per ml of transcobalamin II.

**DISCUSSION**

Transcobalamin II has been isolated in homogeneous form for the first time after being purified 2 million-fold relative to human plasma. Affinity chromatography on vitamin B12-Sepharose was the crucial purification technique employed and resulted in a 24,000-fold purification of transcobalamin II. The fact that this technique has been applicable to the isolation of the granulocyte vitamin B12-binding protein (14) as well as transcobalamin II suggests that it may be of general value in isolating other vitamin B12-binding proteins.

Plasma fractions containing transcobalamin II facilitate the uptake of vitamin B12 by a number of different types of cells (9-12). The availability of homogeneous transcobalamin II allows for new experiments to elucidate the mechanism of protein facilitated cellular uptake of vitamin B12. Preliminary experiments indicate that our final preparation of transcobalamin II does facilitate vitamin B12 uptake by confluent cultures of human diploid fibroblasts. Thus, vitamin B12 bound to transcobalamin II is taken up by fibroblasts in significantly greater amount than either unbound vitamin B12 or vitamin B12 bound to the granulocyte vitamin B12-binding protein. This result indicates that our final preparation of homogeneous transcobalamin II retains its functional ability as well as its ability to bind vitamin B12.

Studies using crude preparations of transcobalamin II have indicated that this protein contains a single vitamin B12-binding site (18) and has a molecular weight of 36,000 to 38,000 (17, 18) when determined by gel filtration. Our studies using homogeneous transcobalamin II also indicate that the protein has a single vitamin B12-binding site, but our studies demonstrate a molecular weight of approximately 60,000 when measured by gel filtration, sedimentation equilibrium ultracentrifugation, and amino acid analysis. We have determined that transcobalamin II is a dimer consisting of 1 approximately 38,000 molecular weight subunit and 1 approximately 25,000 molecular weight subunit. Additional gel filtration experiments suggest that the 2 subunits may dissociate under certain conditions or that the protein interacts with Sephadex thus resulting in an apparent molecular weight of 38,000 based on the elution profile of protein-bound vitamin B12. It is of interest that studies using crude transcobalamin II yield gel filtration molecular weight values greater than 38,000 for this protein under certain conditions (18-20) and that partial purification or high salt concentrations, or both, are required to obtain transcobalamin II in its 36,000 to 38,000 molecular weight form (2).

Transcobalamin II has a number of properties in common with the granulocyte vitamin B12-binding protein (14), but the two proteins also differ in a number of respects. Similar properties include: (a) both proteins appear to have single vitamin B12-binding sites and have molecular weights close to 60,000. (b) The presence of vitamin B12 is required to obtain maximal vitamin B12-binding activity when the proteins are renatured from 7.5 M guanidine HCl. (c) Sulfhydryl compounds are deleterious to the renaturation of both proteins. (d) Neither protein contains any demonstrable free sulfhydryl groups. (e) When vitamin B12 is bound to either protein there is a general enhancement of the vitamin B12 spectrum above 300 nm.

Differences between transcobalamin II and the granulocyte vitamin B12-binding protein include: (a) transcobalamin II contains one 38,000 molecular weight subunit and one 25,000 molecular weight subunit, whereas the granulocyte vitamin B12-binding
protein appears to consist of a single polypeptide chain. (b) Transcobalamin II is eluted from vitamin B_{12}-Sepharose at lower concentrations of guanidine HCl and more rapidly than is the granulocyte protein. (c) Transcobalamin II is not a glycoprotein, whereas the granulocyte vitamin B_{12}-binding protein contains 33% carbohydrate. (d) The amino acid compositions of the two proteins are very different with major differences in their content of histidine, arginine, proline, alanine, leucine, and methionine. (e) When vitamin B_{12} is bound to transcobalamin II the 361 nm spectral peak for unbound vitamin B_{12} shifts to 364 nm. No shift occurs when vitamin B_{12} is bound to the granulocyte vitamin B_{12}-binding protein. (f) The difference spectra between the individual protein-vitamin B_{12} complexes and unbound vitamin B_{12} are quite different and suggest that the vitamin B_{12}-binding sites for the two proteins are not the same. (g) Transcobalamin II appears to facilitate the uptake of vitamin B_{12} by human diploid fibroblasts, whereas the granulocyte vitamin B_{12}-binding protein does not.

The differences between the amino acid and carbohydrate compositions of transcobalamin II and the granulocyte vitamin B_{12}-binding protein are compatible with the immunological differences that have been observed (2). We have previously summarized the immunological and other similarities between the granulocyte vitamin B_{12}-binding protein and transcobalamin I (14), and, on the basis of the differences between the former protein and transcobalamin II, it appears very unlikely that transcobalamin I and transcobalamin II are structurally related or that transcobalamin II is converted to transcobalamin I as has been suggested (12, 21).

Puutula and Gräsbeck (22) have recently purified transcobalamin II to the point where only approximately 60% to 70% non-vitamin B_{12}-binding protein was present. Only 60 µg of protein were obtained because of the low yield concomitant with a long series of conventional purification techniques. Despite this small amount of protein a number of physical studies were performed and several of these demonstrated different results than we have obtained.

Using the phenol sulfuric acid method (23) they obtained a 13.6% neutral hexose content for their final preparation of transcobalamin II. We have detected no carbohydrate residues using larger quantities of protein for analyses that have included amino acid analysis for amino sugars and a gas-liquid chromatographic method of carbohydrate analysis as well as the phenol sulfuric acid method. The most likely explanations for this discrepancy are that the 60% to 70% contaminating protein present in the final preparation of Puutula and Gräsbeck contained carbohydrate or that small fragments of Sephadex were present in their final preparation since gel filtration was used extensively in their purification scheme.

Puutula and Gräsbeck obtained a molecular weight for the transcobalamin II-vitamin B_{12} complex of 20,000 to 30,000 by sedimentation equilibrium ultracentrifugation in which the cells were scanned only at A_{280}. We obtained a molecular weight of 53,000 using the same technique and obtained similar values regardless of whether the cells were scanned at A_{280} or A_{260}. There are several possible explanations for the molecular weight discrepancy and these include: (a) the 60% to 70% contaminating protein present in the final preparation of Puutula and Gräsbeck may be responsible since their cells were scanned only at A_{280}. (b) The 2 transcobalamin II subunits that we have demonstrated may not have been associated during their sedimentation equilibrium experiments. (c) If one of the 2 transcobalamin II subunits is capable of binding vitamin B_{12} alone, then Puutula and Gräsbeck may have isolated this subunit alone.

The latter possibility could conceivably also account for the third difference between their work and ours which concerns the fact that they did not observe a spectral shift in the vitamin B_{12} peak at 361 nm when the vitamin is bound to transcobalamin II, whereas we observed a shift to 364 nm. It is possible that the presence of both transcobalamin II subunits is required for the 361 nm → 364 nm shift and that this would not be observed if only 1 subunit was present. This question should be resolved when we complete our attempt to isolate the 2 subunits separately and study the role of each subunit in vitamin B_{12} binding and the effect that each subunit has on the vitamin B_{12} spectrum.

Acknowledgments—The authors thank the American Red Cross National Fractionation Center for providing Cohn Fraction III of human plasma, and Dr. David Alpers for performing the carbohydrate analyses using gas-liquid chromatography. We thank Carmelita Lowry and Susan Holmes for their assistance in performing molecular weight determinations. We also thank Carol Mehman and Roni Rosenfeld for their assistance. We also thank Dr. Ralph Gräsbeck for a copy of his manuscript (22) prior to its publication.

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*J. Biol. Chem.* 1972, 247:7709-7717.

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